

A judgement fit for prime time

Last week's verdict on a celebrated case of scientific misconduct describes a series of misconceived accusations, ill-considered evidence and faulty judgements. But researchers should hesitate before taking too much comfort from the outcome.

AMONG those who worry about the public understanding of science are a few naive souls who believe that things would get better if researchers featured in soap operas. But when science itself becomes high drama, with intellectual cut-and-thrust tinged with soapily charged emotions, the sang-froid of television executives has been known to turn, well, lukewarm. Now, as a worthy successor to 'Life Story' (a superb dramatization of the Watson-Crick discovery), comes a story-line from the US Department of Health and Human Services (DHHS): 'Research integrity adjudications panel, subject: Thereza Imanishi-Kari'.

Equivalent in length to three-quarters of an issue of *Nature*, the document (see page 719) represents the climax of a ten-year saga in which the perceived roles of individuals and organizations have shifted from the villainous to the heroic, and vice versa. Those involved are gifts to script-writers: a congressman apparently determined to show that scientists cannot police themselves; a Secret Service forensic department instructed by him to spend on laboratory notebooks more time, as it turns out, than they have ever spent on a threat to a president or on an illicit nuclear arms transfer; an accuser whose past motivation and character as a scientific colleague are open to question; an investigative body — the Office of Research Integrity (ORI) of the National Institutes of Health (NIH) — whose capacity for objective and intelligent judgement now stands implicitly condemned; a Nobel prizewinner who felt forced to resign from a university presidency over the affair, withdrew the disputed paper of which he and the accused were co-authors, and then retracted his withdrawal; and the immunologist whose reputation now stands triumphantly restored: Dr Imanishi-Kari.

The new judgement follows others reached less legalistically by the Massachusetts Institute of Technology, Tufts University, the NIH and the US Attorney's Office of the District of Maryland, all of which backed the accused against allegations by Margot O'Toole that Imanishi-Kari had falsified data to support the conclusions of a paper published in *Cell*. And it appears to vindicate those who warn against outsiders becoming involved in the policing of scientific conduct: witness Secret Service conclusions reached "without an appreciation of the bigger picture".

That conclusion would be premature. There are fierce debates in progress in the United States about how academic institutions might in the future be required to change their approach to scientific misconduct (see the latest counter-attack launched by Kenneth Ryan, chairman of a congressionally mandated panel set up to look at the issue, reported on page 719). The secretive way in which universities as distinguished as Harvard handle misconduct investigations (see *Nature* 377, 569; 1995) does not inspire confidence that publicly funded academics are subject to sufficient sanctions to deter wrongdoing.

But the biggest question now hangs over the ORI. Scandalously, it was the DHHS Research Integrity Adjudications Panel, the final point of appeal in such cases, that provided the first opportunity for Imanishi-Kari to confront the evidence and witnesses on which ORI based its 1994 judgement that she had committed misconduct, barring her from receiving federal funds for 10 years. That evidence, and the way in which it was deployed by the ORI, is repeatedly dismissed in the adjudication. But this was not explicitly

a trial of the ORI, and the roles of individuals in the ORI are not discussed. That is a major gap in the script that needs to be filled by the NIH and the DHHS, and conclusions about the ORI's future reached, before some confidence in the integrity of public judgements of misconduct can be restored. □

New dawn in Italy?

The new Italian research minister should be the first for some time to make a difference.

WE have seen it all before: a new Italian research minister pledging to introduce dynamism into the country's bureaucratic and over-centralized research and university systems, where, for decades, favouritism and acceptance of mediocrity have reigned hand in hand. Italy's scientists have watched a steady procession of research ministers ineffectively come and go with every short-lived government. They might be forgiven for wondering why Luigi Berlinguer should succeed in bringing about the necessary revolution where others have failed.

But there is something different this time. This government is not simply a reshuffle of the same old people, and parliament is awash with new faces. Old networks appear to have been broken, making real change possible. That is an opportunity to be seized.

As a seasoned politician, Berlinguer knows that he can make progress not only by creating new laws but also by pursuing the implementation of old ones. In that spirit, his main target is a law that has been gathering dust for six years, which would decentralize the CNR, Italy's research council which runs 350 institutes and research centres. Allowing institutes to develop their own rules and distribute their own budgets will help them on the road to greater efficiency. Because Berlinguer is prepared to make important personnel changes, he is attacking the huge inertia within the CNR, from its top rungs of political appointees to its lower rungs of ill-informed civil servants, which has blocked that road for so many years.

Berlinguer should not shy away from new laws to deepen the reforms. The CNR's arcane system of committees, which decide how resources should be allocated between its research institutes and university centres, is in dire need of such change and should be opened up to the scrutiny of the wider international community. The electoral system by which committees are appointed serves only to maximize the tendency for members to defend their disciplines and be nice to their peers, at the expense of selectivity and tough decisions of priority. As a result, for example, no research institute is ever closed down, whatever its level of productivity.

Berlinguer must create a truly independent system of evaluation to help the CNR to decide more dispassionately where to concentrate its money. He should consider introducing something similar to the Max Planck Society's *Fachbeirat* system, where advisory groups comprising both national and international experts are appointed to each institute to advise it on its research plans and to submit an independent assessment of its achievements to the central headquarters. □



Clearing of researcher in 'Baltimore affair' boosts demand for reforms

Washington. Ten years after charges of scientific misconduct were first levelled against her, Thereza Imanishi-Kari, an immunologist at Tufts University Medical School in Boston, has been cleared of wrongdoing by the appeals board of the US Department of Health and Human Services (DHHS).

The board's decision, released in Washington last Friday, overturns a highly publicized ruling in 1994 by the Office of Research Integrity (ORI) of the National Institutes of Health (NIH), which found Imanishi-Kari guilty of scientific misconduct and fraud (see *Nature* 372, 391; 1994).

This followed allegations that data in a laboratory notebook concerning experiments on gene expression in transgenic mice, carried out between 1984 and 1986 at the Massachusetts Institute of Technology (MIT) in collaboration with David Baltimore, then head of the MIT's Whitehead Institute, appeared to contradict data published in a paper in the journal *Cell* at the end of this period.

The Imanishi-Kari affair has become more than a solitary case of alleged scientific

misbehaviour; for many, it now represents the worst aspects of government handling of scientific misconduct allegations. By overruling the ORI verdict, the appeals board has opened the door to calls for reform of the current system for dealing with allegations of scientific misconduct.

But the most immediate impact of the appeals board ruling is to clear Imanishi-Kari herself. "This is absolutely fantastic, but I haven't had time to enjoy it," she said the day after the verdict. "I am so pleased that there are some honest people who can make a fair judgement."

Imanishi-Kari currently works as a research associate in the department of pathology at Tufts. She held a faculty appointment at the university until the ORI verdict forced the university to strip it from

her, although university officials have allowed her to continue her laboratory research pending the result of the appeal.

The case began in 1986, when the allegations against her were made by Margot O'Toole, a postdoctoral research fellow in Imanishi-Kari's laboratory. O'Toole took her concerns to both MIT and Tufts University, which was considering Imanishi-Kari for a position at the time. Officials at both institutions decided that the dispute was of a kind "not uncommon in science".

O'Toole's persistence in questioning the *Cell* paper led the NIH to set up a panel of inquiry. This found errors in the paper requiring correction, but no evidence of "fraud, misconduct, manipulation of data, or serious conceptual error". But by then the House of Representatives, through John Dingell (Democrat, Michigan), chairman of the oversight and investigations subcommittee of the Committee on Energy and Commerce, had opened hearings into the matter.

Baltimore's spirited public defence of Imanishi-Kari at the hearings led to the case becoming informally known as "the Baltimore affair", even though Baltimore himself was never accused of misconduct. (His intervention also cost Baltimore the presidency of Rockefeller University.)

Dingell, a tireless critic of the 'self-policing' policy of the US scientific community, enlisted the US Secret Service to conduct forensic studies of some of Imanishi-Kari's laboratory notebooks, including analysis of their paper and ink, as well as the radiation counter tapes they contained, to establish the dating of relevant experiments.

According to the Secret Service forensic experts testifying before the appeals board, there were some inconsistencies that could be construed as fabrication of data. But they were quick to add under questioning that they had no previous experience in analysing laboratory notebooks.

Nevertheless, ORI eventually found Imanishi-Kari guilty on 19 counts of scientific misconduct and banned her from receiving federal research grant money for ten years. Imanishi-Kari appealed against the verdict to the DHHS departmental appeals board in November 1994, and the verdict was finally delivered last week.

The panel appointed to hear the appeal has now ruled that "no debarment be imposed" and that "no other administrative actions should be taken", effectively closing the case for good.

But the publicity that has surrounded the case means that its fallout is likely to be ►

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Imanishi-Kari: likely to seek reinstatement.

Seth Resnick

Problems of integrity are 'pervasive'

Washington. The chairman of a congressionally-mandated commission on misconduct in research has hit back at critics who claim that the recommendations of a recent report by the panel are too draconian, and has warned that problems of integrity are now "pervasive" in US science.

Kenneth Ryan, professor emeritus at Harvard Medical School and chairman of the Commission of Research Integrity (CRI), says that its proposals must be acted on because the scientific community has ignored a report published in 1992 by the National Academy of Sciences, which recommended extensive self-regulation of scientific conduct.

He also predicted that Donna Shalala, the health secretary, will implement most of the CRI's recommendations, despite the protests of scientists and groups such as the Federation of American Societies for Experimental Biology (see *Nature* 381, 639; 1996).

"It isn't the commission that is on trial here — it is the scientific community," he told a seminar in Washington last week organized by George Washing-

ton University and the American Association for the Advancement of Science.

Ryan hit out at scientists who have attacked the 12-man panel for its failure to include "distinguished" scientists. "That's such an easy thing for scientists to say," said Ryan. "The twelve were chosen to represent the public interest, not scientists' interests."

Congress set up the panel in 1993, and Shalala is now considering its recommendations. "I'm very, very cynical about the scientists who are protesting so much" about the CRI findings, Ryan said. "I'd like to see them stand up for what was in the National Academy of Sciences report."

Speaking before the final verdict, delivered four days later, in the long-running case involving David Baltimore, the Nobel prizewinner, and Thereza Imanishi-Kari (see above), he also warned scientists not to draw comfort from the result. "If Imanishi-Kari is exonerated, I can see a lot of people saying that there is 'no problem'. But there is more to [scientific integrity] than these high-profile cases."

Colin Macilwain

► felt for some time. Joseph Onek, for example, the lawyer for both Imanishi-Kari and Baltimore, says that the appeals board's ruling is a "great thing for Dr Imanishi-Kari, but I also think it is a great thing for science". He adds: "I hope that this case will now lead the government and scientific community to reassess how these scientific misconduct disputes are handled, and try to figure out ways to resolve them more promptly and fairly."

ORI, never popular among scientists, is now likely to lose even more support. Indeed, the heaviest criticism in the appeals board's ruling was levelled at the integrity office, saying that much of the evidence it presented was "irrelevant, had limited probative value, was internally inconsistent, lacked reliability or foundation, was not credible or not corroborated, or was based on unwarranted assumptions".

The appeals board ruling comes at a time when proposed new scientific misconduct regulations are being hotly debated both within DHHS and in the scientific community at large. How the department should respond to a congressionally mandated report on scientific misconduct is being considered by Shalala and her staff (see *Nature* 639, 381; 1996 and previous page).

In addition to ORI, another likely casualty of the appeals board ruling is O'Toole, now a researcher at the Genetics Institute, a private biotechnology company in Cambridge, Massachusetts. "The [board] has had the same initial reaction everybody has had: they can't believe that what I said happened, did in fact happen," says O'Toole. "But since they have tossed out the evidence, their conclusions are not surprising."

The appeals board called O'Toole's interpretations of some events "improbable and unwarranted", and parts of her testimony "not credible". But O'Toole takes issue with such statements. "From the beginning, I have always told the truth, with the full expectation I would be branded a liar for doing so," she says. "The miracle was that, without exception, every scientist who examined the evidence, eventually — and reluctantly — came to the conclusion I was telling the truth."

The panel said it was important that for 'whistleblowers' to be protected from adverse consequences. But it also warned that they should not get too heavily involved in a subsequent investigation. "Such involvement can compromise both the ability of the investigators to maintain objectivity, and the ability of the whistleblower to avoid becoming too vested in the outcome," it says. "We think that happened here."

Imanishi-Kari says that her first priority now is to seek reinstatement of her faculty position at Tufts University, a request which is likely to be granted by the university, which has been "very supportive" through the whole affair. "Then I will be back to the usual," she says. "Trying to get funding for my research."

Fintan Steele

Italian minister promises to cut research bureaucracy

Rome. Luigi Berlinguer, Italy's new research and education minister, promised last week that research will be one of the new government's highest priorities — even though pre-election pledges of increased funding may have to wait for improvements in the economy.

After one month in office, Berlinguer says he is keen to increase the cost-effectiveness with which Italy spends its research budget, in particular by requiring its notoriously bureaucratic research and university systems to increase their efficiency.

But he wants to do this as far as possible without introducing new laws into areas which, he says, already suffer from excessive legislation. At the same time, Berlinguer plans to introduce radical changes to the recruitment of university faculty members, raise support for scientists in the relatively impoverished south of Italy, and push for increased joint research funding by the European Union (EU).

Berlinguer is a member of the Democrat Party of the Left (PDS) — the successor to the former communist party — with long parliamentary experience. He was even made research minister in Carlo Ciampi's interim government in 1993, but his appointment lasted only a few hours before the PDS pulled out of the government.

One of his major targets, he says, is Italy's National Research Council (CNR), which funds around 350 research institutes and university centres. Scientists have long been frustrated by extensive delays in allocating CNR funds, and what they claim to be a general mismanagement of resources.

Many argue that the roots of such problems lie in the highly centralized organization that has existed since the research body was first established by Benito Mussolini in the early 1920s. Berlinguer says that he plans to push for the full implementation of a law drawn up at the end of the 1980s by Antonio Ruberti, the former research minister, giving institutes sufficient autonomy to make their own internal regulations and control their own budgets.

At the same time, he plans to speed up grant review processes and simplify administrative procedures carried out through CNR's central headquarters, where staff numbers will be cut by relocating administrative positions to individual institutes.

A similar approach will be taken to the

CNR's 15 scientific advisory committees, which Berlinguer says are both too numerous and too large. Broader reform of the committee system, which would require an act of parliament, may follow later.

One area where he is already planning legislative action is reform of the controversial system of appointing university staff through national competitions — or *concorsi* — widely criticized for giving considerable weight to the personal connections of candidates (see *Nature* 378, 228; 1995).

Attempts by previous ministers to change the system have been unsuccessful, largely because of resistance from parliamentary professors who have themselves benefited from it. Although the parliament is still dominated by university professors, Berlinguer — who is himself professor of law at the University of Siena — hopes that fresh blood will judge more favourably his own radical reform proposals, the details of which will be announced shortly.

Another of Berlinguer's priorities is to find ways of recruiting more young scientists in southern Italy, where the proportion of researchers in the population is six times lower than in the industrialized north.

Various programmes to improve the science base of the south have been introduced by previous governments over the past few years, but he says that there is little to show for the IL1,000 billion (US\$640 million) invested so far. "The idea that money could be rained down on the south and that would be enough to make things work was ill-conceived. In future, it must be clear that the infrastructure is there to support research programmes."

The programmes will be relaunched, but with more checks and controls to ensure that investments are better protected. In addition, says Berlinguer, a recruitment drive will be introduced for young scientists, offering a large number of scholarships and temporary research contracts.

Even though Italy cannot afford to raise its relatively low level of investment in research — despite being the fourth largest economy in Europe, it spends only 1.3 per cent of its gross national product on research and development, barely half that of many other European countries — it will still propose an increase in the EU's fifth Framework programme of research, which will run from 1998 to 2002.

Berlinguer justifies this on the grounds that Europe can only compete effectively with Japan and the United States if member states join forces as extensively as they can, although others point out that Italy would hope to be a net beneficiary of any increased spending on Framework. **Alison Abbott**



Berlinguer: 'south needs more science'.

Financial blow slows wind-power projects

Boston. Prospects for the development of wind power as a major energy source received a setback at the end of last month when Kenetech Windpower, Inc., the largest developer of 'wind farms' in the United States, filed for bankruptcy protection under Chapter 11 of the US bankruptcy code.

The move jeopardizes some large wind-power projects that the company, based in San Francisco, California, was pursuing in California, Maine, Oregon, Washington and Wyoming. Kenetech has not yet announced whether it intends to liquidate all of its assets or reorganize. It is trying to find other developers to take over these projects, which make up about half of the large wind installations planned in the United States.

"It will be difficult for them to find buyers in the current energy market," says Daniel Sosland, an attorney with the Conservation Law Foundation, one of four groups that obtained environmental concessions from Kenetech during negotiations over plans for a proposed wind farm in northern Maine.

Sosland says that the drop in electricity prices over the past five years has been the "single largest factor" contributing to Kenetech's demise. But several other factors played a role, he says, including mechanical problems with the company's wind turbines and a series of lawsuits over warranty claims and other issues.

According to Randall Swisher, executive director of the American Wind Energy Association, an industry trade group, part of the blame rests with inconsistent government policies. He argues that Kenetech may have lost half a billion dollars worth of business because of a decision by the Federal Energy Regulatory Commission in 1995 which struck down an agreement with the

state of California that had been five years in the making. Swisher cites other decisions as examples of shortsighted policies, including a recent vote by Republicans in the House of Representatives to eliminate tax incentives for users of wind-energy systems.

But Swisher still maintains Kenetech's failure was "the failure of a single company and not of the domestic wind-power industry in general". Sosland agrees up to a point, although he acknowledges that US wind companies have lost their lead in this field to European companies.

Moreover, the level of private and public investment in such technology in the United States is falling, a trend that he and other clean-power advocates consider alarming. "At some point, the current energy glut will dry up," Sosland argues. "If we let the renewable energy industry collapse in the meantime, it won't be around when we need it."

Steve Nadis

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An ill wind: falling electricity prices have added to Kenetech Windpower's woes.

Quick launch urged for Cluster 'spare'

Munich. The scientific advisory committee of the European Space Agency (ESA) proposed last week that the spare unit of the four-satellite Cluster mission should be prepared for launch. The original mission was destroyed when the first qualification flight of ESA's Ariane-5 launcher failed earlier this month (see *Nature* 381, 541; 1996).

But a vote on the proposals was deferred by the Science Programme Committee, which comprises delegates from ESA's 14 member states, and to which the advisory committee reports, as France wants the whole mission to be relaunched. A final decision on the next step for Cluster will be

taken next week.

The Space Science Advisory Committee (SSAC) argues that the spare unit could be reassembled and equipped with instruments at a relatively low cost — ECU30 million (US\$37 million) — which would be within the ESA science programme's contingency funds. This would mean that its costs would not eat into the budget of the programme's other tightly planned missions.

It would also mean that ESA would be able to fulfil international commitments. The possibility of three-dimensional analysis of the electric and magnetic environment of the Earth as it interacts with the solar wind, carried out by the four satellites flying in formation, would be lost.

But the launch of a single unit into the planned orbit — the so-called polar cusps where solar particles can enter the magnetosphere — would at least mean that ESA could maintain its commitment to the wider global terrestrial physics programme, the Interagency Solar Terrestrial Programme, a series of coordinated missions that take measurements in different orbits.

This plan should be implemented immediately, says SSAC, before research and operational teams are broken up. Quick development of the single unit would allow launch on a further Ariane-5 qualification flight, probably next summer.

But France, which had several instruments on Cluster, wants ESA to look more closely at the possibility of relaunching the whole mission, thus saving its original scientific aims — although almost certainly disrupting other space science missions — at a cost of around ECU300 million.

Alison Abbott

Europe narrows patent gap with America

Munich. The number of patent applications filed last year by European countries at the European Patent Office (EPO) in Munich increased significantly faster, at 8.1 per cent more than in 1994, than those filed by the United States, which increased by 5.3 per cent. Applications from Japan fell for the first time, by more than one per cent.

Europe's innovative push — primarily from Germany and France — was largely responsible for the 5.5 per cent increase in patent applications filed through the EPO. Figures for the first half of this year indicate the trend is accelerating.

But Ingo Kober, the president of the EPO, warned in presenting the organization's 1995 annual report last week that although the gap with the United States appears to be narrowing, Europe still has a lot of catching up to do. In 1995,

applications from the United States accounted for 29 per cent of the total.

The number of applications in medical and veterinary technologies rose by 7 per cent during 1994. But the highest rate of increase was in patents in computing (11.3 per cent) and electronic communication technologies (12.5 per cent), this time predominantly because of increased applications from the United States and Britain.

Kober repeated a promise to use the surplus accumulated by the EPO, now over DM176 million (US\$276 million), to cut the high costs of patent applications. This cost has been widely criticized as discouraging innovation in Europe (see *Nature* 375, 270; 1995). Kober says that the EPO is a service organization, and should not be generating such a high surplus.

Quirin Schiermeier

Activists seek retirement home for chimps

Washington. Animal rights groups in the United States have proposed the setting up of a National Chimpanzee Sanctuary, paid for jointly by the National Institutes of Health (NIH) and charitable contributions, to enable the country's estimated 1,500 research chimpanzees to 'retire' in comfort once they are no longer needed — if they are needed at all.

The groups have made the proposal as the National Research Council (NRC) embarks, at the request of NIH, on a study of what to do with the chimpanzees, which can live to the age of 55. Boosted in number by a breeding programme which the NIH started in 1986, many hundreds of the chimps are no longer used for research.

The chimps cannot be returned to the wild, and public opinion would be against putting them down. So they continue to languish in single cages in half-a-dozen centres.

Directly or indirectly, the NIH pays most of the \$10 million a year that they cost to keep.

A panel of the NRC, chaired by Dani Bolognesi, director of the Duke Center for AIDS Research, North Carolina, has been asked to set out options for what to do with the chimps and who should pay for their care. At an initial public meeting of the panel in Washington last week, hopes were high that animal rights groups and biomedical researchers can, for once, find common ground.

But their unlikely alliance may founder on the rock of finance. One activist — Christine Stevens of the Animal Welfare Institute — suggests that NIH should set aside \$100 million to care for the animals indefinitely. NIH managers accept some responsibility for long-term care, but want to reduce their existing costs.

Michael McGehee, a lobbyist for animal

rights groups, says that legislation to establish their proposed sanctuary will be introduced in Congress next month. The proposal would require the government to match charitable donations to run the sanctuary of up to \$5 million a year. The legislation's chances are slim, but a cost-shared sanctuary remains the most likely destination for 'retired' research chimpanzees.

The panel was told that successful sanctuaries exist in Africa to look after orphan chimpanzees. According to Don Buford, chief executive of the Jane Goodall Institute, which runs them, they could serve as a useful model for US sanctuaries.

But he warns that the return to Africa of US research chimpanzees, many of which are infected or potentially infected with diseases such as HIV and hepatitis B, would be "fiercely opposed" by African governments. The infectious diseases will also complicate

Russian institutes fear lost deposits

Moscow. Russia's presidential election campaign has brought relief to cash-starved government research laboratories. Following a promise by President Boris Yeltsin, all sums owed by the state have been transferred to the banks acting for research institutes and laboratories.

But just as these clouds over Russian science were beginning to disappear, a new misfortune has arrived. Most scientific institutions have been keeping their money in small commercial banks, as larger banks have been reluctant to handle the relatively modest sums involved. But one of these has now stopped paying out on deposits, giving rise to fears that substantial research funds may be lost.

A number of institutes of the Russian Academy of Sciences had deposited their funds with the Balchug bank in Moscow. Last month, for example, both the Russian Ministry of Science and Technological Policy and the Russian Foundation for Fundamental Research transferred to the Balchug bank funds for several institutes, including the Institute of Molecular Biology and the Institute of Gene Biology.

But when the institutes started sending payment orders to their bank, even though requests for small transfers were processed normally, it soon became apparent that others were being ignored. To make matters worse, many of the institutes used their accounts with the bank to hold money allocated for the national scientific programmes, due to be distributed to a range of laboratories.

The sum of 750 million rubles

(US\$150,000) deposited by the Microbiology Institute, for example, includes 400 million rubles allocated to the 'Human Genome' project and more than 100 million rubles allocated to a national project on the 'material base for biology studies development'.

As a result, dozens of Russian research institutions have been unable to withdraw money allocated to them by the Russian government. "As soon as we found out that the Balchug bank was not processing our payment orders, even though it did not say it was bankrupt, we informed both the Ministry of Science and Technological Policy and the Central Bank of Russia, which is meant to have special reserve insurance funds for such occasions," says Alexander Zelenin of the Molecular Biology Institute.

"But we did not get a reply, and have had to contact another bank, Menatep, which is large and reliable, but inconvenient for us in many ways." Zelenin hopes that, after the elections, the authorities will find time to sort out these problems and to return to scientists the money given by the government during the election campaign.

According to Oleg Baranov, vice-president of the Balchug bank, its current difficulties are the result of loans not being repaid in the light of economic uncertainties over the forthcoming election. Its major debtor is a joint stock company, Bulchug, which links together about a dozen commercial operations and has withdrawn its support for the bank — even though it is its main shareholder.

Carl Levitin

Listen. They experiment on us, put us in solitary confinement for years, and then this. Human Compassion's just got to be caused by a lentivirus!



any solution which releases the animals from solitary confinement in laboratory cages.

Returning the chimpanzees to their natural habitat is also out of the question; experts agree that they would not survive. Killing them is not illegal, as the chimpanzee is not listed as an endangered species in the United States. But, as Louis Sibal, director of the office of laboratory animal research at NIH, says, "there is an unwritten assumption that euthanasia is not acceptable."

The usefulness of the chimpanzees for AIDS research has not matched the expectations of the NIH when it began its breeding programme. The result is a growing surplus of ageing animals, which either the NIH or the public will have to care for.

The first task of the panel will be to define the issue adequately: no-one really knows the precise size of the surplus, or its cost. (The \$10-million estimate is based on the common assumption that the animals cost between \$15 and \$20 a day to care for in their current laboratory environment). It will issue its report later in the year.

Colin Macilwain

Head of Indian nuclear agency loses job after going critical

New Delhi. The head of India's nuclear watchdog agency, A. Gopalakrishnan, who recently raised questions about the safety of the country's ageing nuclear power plants, has paid the price for being forthright. Although he could have continued as head of the Atomic Energy Regulatory Board (AERB) for another three years, the government last week refused to renew his contract, and replaced him with his junior.

Gopalakrishnan's unceremonious exit has brought into the open a long-standing conflict between AERB and the Department of Atomic Energy (DAE), which presides over the country's nuclear research laboratories and power stations, and whose activities the board is supposed to regulate.

In a scathing attack that has embarrassed both DAE and the government, Gopalakrishnan claimed that the DAE was obsessed with secrecy and intolerant to criticism. He said he was leaving in protest at the way the DAE had ignored the board's recommendations for improving the safety of nuclear facilities.

"The DAE wants the government and the people to believe that all is well with our nuclear installations, but I have documentary evidence to prove that this is not so," he told the *Times of India*.

The secretary of DAE, R. Chidambaram, said the government has a right to renew or cancel the job contract. He argued that there is no serious safety problem with India's nuclear plants and denied charges that DAE was unresponsive to the board's recommendations.

There has been friction between the two agencies ever since Gopalakrishnan, a nuclear engineer trained in the United States, took charge of AERB in 1993 and began to try to increase its effectiveness.

But the real trouble started three months ago when AERB brought out a report listing as many as 150 safety issues — 20 of them in nuclear power stations and the rest in DAE's other establishments — that it said "needed urgent attention". According to Gopalakrishnan, DAE's response was disappointing.

Specifically, AERB inspectors said that the emergency core cooling systems in three out of five power stations were defective. Tanks storing several thousand tonnes of liquid wastes at the Bhabha Atomic Research Centre (BARC) in Bombay have welding defects and needed repairs.

Gopalakrishnan alleged that BARC failed to act on the board's suggestion that there should be a study of the impact of a possible accident at the 100-MW research reactor in Bombay, five kilometres from residential areas.

K. S. Jayaraman

Japan protests at charge of 'dumping' supercomputer

Tokyo & Washington. Japan's Ministry of International Trade and Industry (MITI) expressed "grave concern" last week that attempts by the US Congress to block the purchase of a Japanese supercomputer by the US National Center for Atmospheric Research (NCAR), effectively increasing the centre's computing power by a factor of ten, may constitute a violation of international trade rules.

The strong words came in reaction to legislation being considered in the US House of Representatives this week that would deny salaries to any officials of the National Science Foundation (NSF) who approve NCAR's intended purchase of four NEC supercomputers — if it is determined that the Japanese supercomputers are being sold below fair market value.

The amendment was inserted into the NSF's 1997 appropriations bill by two senior Democrats, David Obey of Wisconsin and Martin Sabo of Minnesota, in whose districts the US supercomputer manufacturer Cray Research is based. Their move followed claims by Cray that NEC won the bidding process by 'dumping' below cost, a conclusion supported by a controversial analysis by the US Department of Commerce suggesting that NEC will lose more than US\$100 million on the sale.

Having solicited bids for high performance computers at a fixed price of US\$35 million, NCAR selected Federal Computing Corporation (FCC), a US company whose bid includes four NEC SX-4/32 supercomputers. NCAR, which will use the computers to run complex climate models, says the FCC proposal won out because it exceeded all performance criteria by at least a factor of two, and provided better cost/performance ratios than bids from three other manufacturers, including Cray.

Last month, when it became clear that NEC was going to win the bid, Cray began to claim that NEC was dumping at below

cost. The Commerce Department took up the issue and last week released an analysis suggesting that NEC is making a loss of US\$111.16 million on the sales, the bulk of the loss being due to high research and development costs. But NEC denies it is selling at a loss, and challenges the validity of the Department of Commerce figures.

NEC has refused to disclose its figures. But it did supply a breakdown of its costs to the University Corporation for Atmospheric Research (UCAR), which runs NCAR. In turn, UCAR commissioned an independent review of the FCC/NEC bid from two consultancy companies. This concluded that NEC's bid was not below "fair value".

Tomio Tsutsumi, Japan's deputy minister for international trade and industry, said last week that the legislation now before Congress, seeking to block the deal, "could violate the principle of non-discrimination in government procurement". MITI officials have suggested it violates international trade rules set by the World Trade Organisation.

The Obey-Sabo amendment was scheduled for debate in the House this week. Jim Kolbe (Republican, Arizona), a strong advocate of free trade, last week proposed his own amendment striking the provision from the appropriations bill, and arguing that any allegation of dumping should be handled through a formal Commerce Department investigation, rather than the intercession of Congress. By the beginning of the week, no such investigation had been initiated, although the department has warned the NSF that it has "significant concerns" about the purchase.

If the award to NEC stands, the NSF will still have alienated two powerful members of Congress. In particular, Obey is likely to chair the House appropriations committee if the Democrats regain a majority in November. In comments accompanying their amendment, Obey and Sabo criticize the NSF for looking after its own interests at the expense of US industry.

They also made a veiled threat. While some scientists argue that the needs of research should take precedence over national economic concerns, they wrote, "wisely, NSF directors have chosen to ignore that advice and, as a result, the foundation has been spared the deep cuts which have been imposed on most other areas of the domestic discretionary budget."

National security is also believed to be an issue in the dispute, with the US Defense Department — which uses Cray computers — reportedly worried that a failure to win the NCAR contract may endanger the company's viability.

Stephen Barker,
Tony Reichardt & David Swinbanks

Cost vs price for NEC supercomputer system (as estimated by US Dept. of Commerce)

	US\$ (million)
Federal Computing Corporation*	19.38
Manufacturing	24.90
Sub total	44.28
Research and development	96.00
Marketing	5.28
Other	1.15
Grand total	146.71
Contract value	35.25
Loss	-111.46

* Federal Computing Corporation (FCC) made the bid and will be the system integrator of the NEC supercomputers.

Source: Nihon Kogyo Shimbun.

BSE maternal transmission results may be released soon

Paris. The UK Ministry of Agriculture, Fisheries and Food (MAFF) is expected to bow to both domestic and European pressure in the near future by releasing preliminary results from a study into the risk of maternal transmission of bovine spongiform encephalopathy (BSE) in cattle.

The study, which is being carried out blind, was set up by MAFF in 1989 and is scheduled to end next year. It is designed to compare the incidence of BSE in the offspring of 315 confirmed cases of BSE with that in offspring from 315 control cows.

But interpreting the study will be more difficult than originally planned, because many animals were fed ruminant protein and could have become infected in that way. The researchers involved say that although this will reduce the statistical power of the study, the latter is still "not too bad". Some experts, however, are concerned that the possible contamination study may leave statistically unresolved the question of whether maternal transmission occurs.

John Wilesmith, the researcher at MAFF's Central Veterinary Laboratory in charge of the study, says that he is making a substantial effort to complete most of it by next month, when he will break the codes hiding information about the group to which each calf belongs, with the aim of publishing the preliminary results in August.

According to Wilesmith, the study was designed so that cattle would be slaughtered on their seventh birthday — a sufficient period to allow the disease to develop — and as most of the cattle were born during the 1988 calving season, they were slaughtered last autumn. It should be possible to

process around 500 brains by next month, says Wilesmith. "It wouldn't be the end point, but it would provide enough for a preliminary publication."

Wilesmith's action follows public calls from Sir Richard Southwood, professor of zoology at the University of Oxford and chairman of an early inquiry into BSE, that the results of the study should be made available early, provided that such a move does not compromise its statistical power. One UK expert says that unless MAFF makes the data available it will be suspected of holding back information. "Secrecy fuels fear," he says.

Indeed, Southwood says that establishing whether or not maternal transmission occurs would help to restore public confidence in the scientific basis for policy decisions about BSE, as well as having practical implications for efforts to eradicate the disease.

Of the 150 brains already processed, 51 have been found to show signs of BSE. Because the study is blind, it is not known to which group these cases belong. But Wilesmith says that this overall incidence suggests that if maternal transmission is occurring, it is at low rates. It is "tending towards zero" and "couldn't be more than five per cent", he says.

If such predictions are borne out by the results of the study, they would confirm field observations suggesting that maternal transmission of BSE occurs at low rates, if at all. According to MAFF, the incidence of BSE in the offspring of confirmed cases of BSE in the field is much the same as would be expected if contaminated feed had been the only source of infection. **Declan Butler**

Venture capitalists invest record funds in US technology

San Francisco. US venture capitalists are spending more than ever on new ideas for technology, according to a recent survey by the accounting firm Coopers & Lybrand, which has found that venture capital spending reached a record level during the first quarter of this year.

The \$2.3 billion was double the sum for the same period last year, and \$150 million more than the previous record of \$2.1 billion, set in the fourth quarter of 1995. "Clearly we are in the midst of an explosion," says James D. Atwell, chairman of the firm's National Venture Capital Group, in the report.

The report says that California enjoyed the most private equity dollars, with 162 deals valued at \$699.4 million. Massachusetts followed, with 79 deals worth \$249.2 million. Software led the top-funded industries, followed by communications, health care, electronics and biotechnology.

Health care showed particular growth, with a 75 per cent increase over the earlier quarter. Coopers & Lybrand say that the medical-related industries are benefiting from the need to serve an ageing population.

The Mayfield Fund in Menlo Park, California, was among the top 10 most active venture capital firms during the quarter. Its newest fund, called the Mayfield VIII, had already funded 23 companies since it closed in October 1995. "It's an unbelievable time," said Grant Heidrich, a partner in the fund.

According to Heidrich, the surge in funding has been driven by three factors; entrepreneurs are presenting excellent ideas to invest in, there is capital available, and the past 18 to 24 months have produced stunning yields for venture companies. In general, he added, Americans are starting to save money in mutual funds and other vehicles, which in turn seek places to invest.

Meanwhile, the cloud over health care and biotechnology investments has lifted with a shift to free market forces in managed care, and a more favourable environment for new products at the Food and Drug Administration.

The record quarter for venture funding followed a record year for initial public offerings of biotechnology companies. During 1995, 61 companies raised \$2.1 billion in stock offerings.

But Heidrich admits that there is some concern that the buoyant market will not last — and cutbacks in research at university level could do serious harm. Five years from now, there may be fewer ideas to invest in, because of a reluctance today to fund bold and risky scientific projects, he says. **Sally Lehrman**

Prion research programme proposed

Paris. The European Commission has proposed setting up a ECU100–150 million (US\$123–185 million) research programme into BSE and other prion diseases. The commission raised the matter at the European Council meeting in Florence last weekend, and is expected to follow this up with a formal proposal in the near future.

The commission is asking that funding for the programme should be additional to existing allocations for research, and should come from other European Union (EU) budgets. A call for proposals would be issued for research to characterize the agents responsible for prion diseases, and to reinforce clinical research on Creutzfeldt–Jakob disease (CJD). The commission is also

seeking to coordinate and strengthen CJD surveillance networks.

Meanwhile, the commission has also agreed to set up a multidisciplinary committee on BSE intended to "ensure that the scientific advice on BSE given to the commission takes into account the widest possible range of expertise". Members of the committee will be chosen by a seven-member steering group, which includes Robert Will, head of the UK National CJD Surveillance Unit.

The new committee will be only consultative, and political power remains in the hands of the agriculture directorate's Standing Veterinary Committee, recently criticized for allowing its judgement to be influenced by political factors (see *Nature* 381, 353; 1996). **D. B.**

Two green bottles leave physicists hanging

Paris. An act of sabotage involving two strategically placed beer bottles has delayed the start of a major new phase of experiments in the Large Electron-Positron Collider (LEP) at the European Laboratory for Particle Physics (CERN) in Geneva, Switzerland.

Physicists at CERN spent last winter upgrading the 90-GeV LEP to transform it into a 161-GeV machine, LEP2, which is expected to open up possibilities for a new series of discoveries. But when the machine was switched on for the first time on 14 June, they were unable to obtain either an electron or a positron beam.

The problem was first put down to teething troubles. But it was quickly found to be the results of a more sinister cause,

namely a blockage somewhere in the 27-km tunnel. Tests narrowed down the location of the obstacle to a 10-metre stretch of the beam pipe, which was dismantled to reveal two empty Heineken bottles, placed 5 metres apart.

While CERN officials joke about Heineken's advertising slogan — that it "reaches the parts other beers can't reach" — they are taking the incident extremely seriously. Police have been called in to investigate and to take fingerprints from the bottles. This is the only way the culprit is likely to be traced, says one CERN official, who adds that it is still not clear whether the act was malicious or simply intended as a prank.

The sabotage is a serious setback, as the

five-day delay it has caused will reduce by almost 10 per cent the time available this summer for running LEP2 at the new energy level. This energy is expected to produce the first pairs of fundamental particles of W^+ and W^- . If the bottles had not been found as quickly as they were, then the impact of the delay would have even more serious.

CERN has tightened security following another incident last year, where a deranged technician cut cables and removed 1,200 components from the LEP accelerator. This sabotage put the accelerator out of action for months (see *Nature* 373, 650; 1995). But officials say that the site is just too big to make it secure against all possible attacks. Declan Butler

China focuses research efforts on grain production

Hong Kong. The Chinese government has announced a 10-billion-yuan five-year programme of support for research projects that can demonstrate an influence on "economic and social development". One-third of the 15 selected projects concentrate on agriculture, including proposals to increase crop yields and promote intensive farming, as well as research into new pesticides and fertilizers.

China's ability to feed itself is a matter of great concern both internally and to the rest of the world. Its commitment to becoming self-sufficient in grain broke down in 1994, when China became a net importer, despite being the world's largest wheat producer. Last August, the Chinese Academy of Sciences predicted that the situation will get worse, with the shortfall of grain reaching 40 million tonnes by the year 2000, and 50 million tonnes — 10 per cent of present consumption — by 2020.

The announcement of the new funding by Deng Nan, vice-minister at the State Science and Technology Commission, appears to be part of a package of measures designed to boost investment in agriculture. It was followed, first, by a decision by China's central government to increase investment in agriculture overall over the next five years, and, two days later, by a speech from Li Peng, the premier, calling for the increased application of science and technology, particularly in agriculture.

At the end of May, more than 1,500 Chinese science representatives and government officials gathered in Beijing for a five-yearly congress at which research priorities for the next five years were agreed. The congress decided that one priority should be agricultural research aimed at increasing crop yields.

The congress was organized by the China Association for Science and Technology (CAST), an umbrella group for about 200 professional bodies and research institutes, including the Chinese Academy of Sciences. It was followed by congresses of individual academies, such as those of science and

through science since 1979. But, he said, the agricultural sector still needs more modern techniques.

The day after the five-day meeting ended, the State Science and Technology Commission announced the formation of the National Corn Engineering Technique Research Centre in Shandong province, intended to import and breed strains of corn seeds and to improve cultivation.

Many institutes around the country study new genetic strains or crossbreeds of grain, and China holds one of the world's largest wheatgerm banks. The government has invested billions of dollars in its 'Spark' research programme, started in 1986 to modernize agriculture through the application of science and technology (see *Nature* 378, 537: 1995). But field testing of new crops is often primitive by Western standards.

For example, the potential dangers of releasing new genetic strains into the environment are said to be often ignored.

CAST claims that, in the past five years, with the help of various science groups and associations, it has disseminated scientific information and techniques to more than 200 million farmers, and it plans to educate another 200 million of China's approximately 800 million farmers over the next five years.

But much of this work, led by the Chinese Academy of Sciences, the Chinese Academy of Agricultural Sciences and the Rural Development Institute of the Chinese Academy of Social Sciences, is not very scientific, and focuses on basic problems such as more efficient fertilizer use — which farmers tend to overuse in a bid to push up yields — better irrigation, and increasing the size of the tiny plots each family at present ploughs.

Elisabeth Tacey

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Taking the strain: modernization of agriculture has become a priority for China's political leaders.

engineering. Although CAST is a non-policy-making body, government officials — including President Jiang Zemin — participated in the congress, giving it a major role in mapping out the future direction of science.

The problem is not only one of growing numbers of people and poorly paid farmers who are deserting rural areas for better paid jobs in the cities, but also the fact that demand for a more varied diet has led to more grain being used to feed livestock. Furthermore, although the central government has made extensive investment in agriculture, local leaders are often keen to sell off land for more profitable industrial production. As a result, rice production has fallen by seven per cent since 1990.

Jiang Chunyun, the vice-premier, told the meeting a 35 per cent increase in the country's agricultural output had been achieved

Lobby group calls for review of key climate change report

London. The Global Climate Coalition (GCC), a US industry lobby group, has called for an "independent review" of the controversy surrounding the rewriting of key parts of the latest report of the United Nations Intergovernmental Panel on Climate Change (IPCC) on the science of climate change.

But the call has been opposed by Ben Santer, a 'lead author' of the report and an atmospheric scientist at the Lawrence Livermore National Laboratory in California, who says there is no need for a review. "The scientific conclusions did not 'substantively change'," as alleged by the GCC, he says. He adds: "I am not going to take part in any 'kangaroo court' when the issue is already decided."

The GCC says that a panel of experts "whose evaluation and objectivity are beyond question" should comment on its recent allegations that the last-minute rewriting of Chapter 8, dealing with the extent of a 'human fingerprint' on global climate change, is against the IPCC's rules (see *Nature* 381, 639; 1996). Santer, as well as IPCC officials, denies the charge. □

Japan's spending aims high

Tokyo. The Council of Science and Technology, Japan's highest body responsible for formulating science and technology policy, has approved a plan to increase government expenditures on science and technology by a factor of about 1.6 by the year 2000. The increase will yield a total expenditure on science and technology over the next five years of ¥17,000 billion (US\$160 billion). But to meet this will require extraordinarily large annual increases in the government's science budget of about 12 per cent a year on average — far above inflation, which typically runs at two to three per cent.

The budget target was included in a programme that the government has been required to map out following the passage late last year of a new basic law for science and technology (see *Nature* 378, 227; 1995). The target is close to one proposed earlier this year by the Liberal Democratic Party (LDP), the strongest party within the ruling coalition government (see *Nature* 381, 6; 1996). □

Japanese carbon emissions up

Tokyo. Japan's carbon dioxide emissions leapt to a record level in 1994, making it unlikely that Japan will meet a commitment to hold emissions to 1990 levels by the end of the decade. According to figures released last week by the Environment Agency, CO₂ emissions totalled 343 million tonnes in 1994, 5.9 per cent higher than in 1993 and 7.2 per cent up on 1990 emissions.

Under the United Nations Framework Convention on Climate Change, agreed in 1992, Japan and other developed nations agreed to hold emissions at or below the 1990 level after the year 2000. But the Environment Agency says this goal will now be hard to achieve unless radical new measures are introduced to cut emissions. □

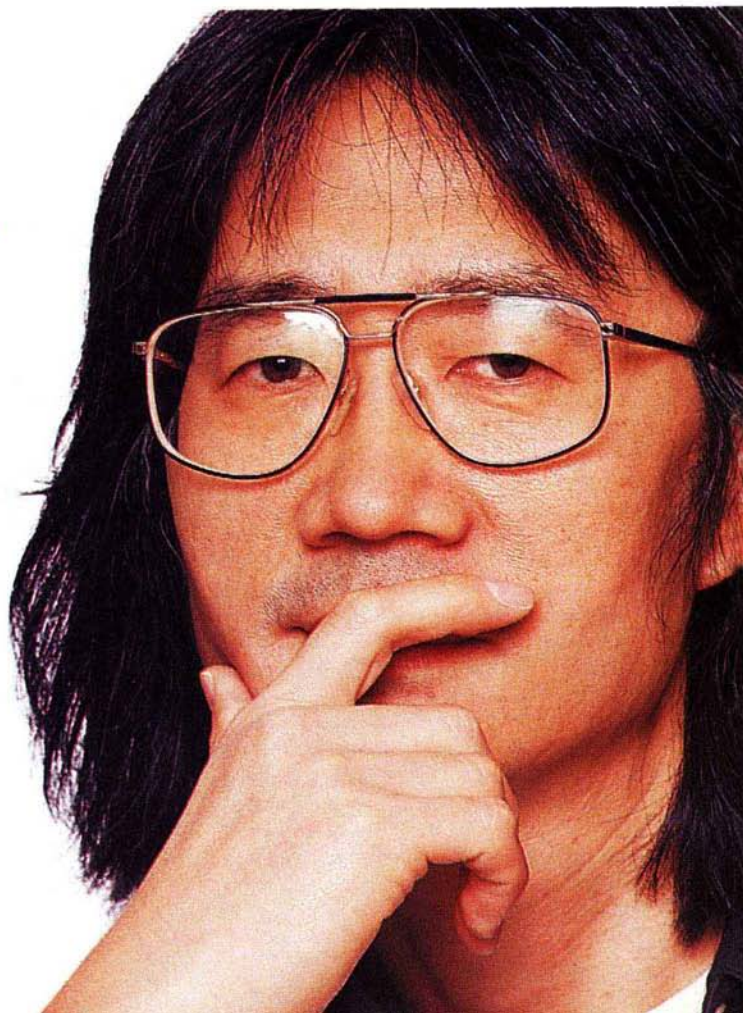
US engineers sack president

Washington. Members of the US National Academy of Engineering have voted overwhelmingly to remove the academy's president, Harold Liebowitz, from office, ending a bitter and protracted struggle for control of the Academy (see *Nature* 379, 761; 1996). The academy's council was informed of the 1,179-to-179 vote on Monday, after an independent auditor had counted the postal ballot.

Liebowitz was immediately removed from office, and the academy's vice-president, Morris Tanenbaum, assumed his duties. The council meets this week to decide the next steps — which will presumably include plans for a special election.

Ousting Liebowitz, in office for less than a year, has not been

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Riddle of the tenth man

SIR — While describing the history of the revelation of the Piltdown hoax, Henry Gee¹ says that the “chemical analyses by Kenneth P. Oakley of the museum [British Museum (Natural History)] showed that all the artefacts were of recent date”. Because the artefacts were of extreme importance and were registered at the museum, it was decreed that they could not be removed from the museum precincts and that no samples could be taken for analysis either within or outside the museum.

The Machiavellian minds of the lawyers, however, decided that it could be considered that the objects could be deemed to be still in the museum if they were in the charge of the curator and if they were returned before nightfall. I had recently developed X-ray fluorescence analysis (XRF) as a nondestructive method of chemical analysis, which was also very rapid and ideally suited if the surface was to be investigated. Indeed, these analyses were probably the very first practical use of XRF for either academic or commercial purposes^{2,3}. (Many other methods of chemical and physical examination using classical techniques were used by the Natural History Museum after it had been discovered⁴ that the bones were not in fact so irreplaceable!)

When mentioning the comparison of the staining on the original Piltdown specimens and the teeth found in Hinton's trunk, Gee quotes Professor Brian Gardiner as accusing Martin Hinton (late of the Natural History Museum) of the fraud, and saying: “But Oakley did not look for manganese. Crucially, analyses of the contents of Hinton's trunk by Currant and Gardiner show that they are enriched in iron as well as manganese — in the same proportions as in the Piltdown specimen.” To quote from my thesis²: “... all specimens were also analysed for manganese which was found to be absent in all cases down to the sensitivity limits of the apparatus. Potassium permanganate staining had been suspected but it was shown that no such treatment had taken place.” It is clear, therefore, that if manganese has been found in the recently discovered “trunk” samples, it is evidence that the two sources are *not* similar.

Gee's report gives a description of how the staining of the bones was achieved by treatment with chromic acid in order to turn the bone apatite to gypsum. It is not clear to me why apatite (calcium phosphate) should be turned into gypsum (calcium sulphate) by treatment with chromic acid. Moreover, during the work carried out in 1953, it was evident that the chromium was associated with potassium ions, and there is little doubt that it was potassium dichromate that was used in the staining process and not chromic acid. Indeed, experiments were

carried out to test this hypothesis using other bone samples; the results gave final products very similar to the original in both chemical analysis and colours. Incidentally, Gardiner claims that the orang-utan mandible was not stained in the same way as the other artefacts, whereas reference to the 1953 thesis shows that its chromium content is very close to the average chromium content of the other eight chromium-stained samples.

I have a list of nine other candidates who have been cited at one time or another as the Piltdown forger. Martin Hinton, whom Gardiner now believes was the perpetrator, is just another to be added to this list. The evidence presented *may* show that he was involved, probably with others, but in no way is it proved. Charles Dawson, a proven fraudster in other spheres, seems a much more likely candidate.

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SIR — Brian Gardiner's contention that Martin A. C. Hinton was the perpetrator of the Piltdown hoax adds another culprit, but he lets Charles Dawson off the hook too easily. He should also consider the chapter on “The Piltdown Perpetrator” in the book *Mysterious Realms* by Joe Nickell with John F. Fischer (Prometheus Books, Amherst, New York, 1992).

Dawson was the one person consistently present at all the Piltdown discoveries. To say he was Hinton's dupe throughout gives Hinton almost omniscient power over Dawson. It is far more likely, given the discovery of Hinton's trunk of coloured bones at the museum, that Hinton was a collaborator, even the junior partner in the affair. For the discoveries at Piltdown ceased with Dawson's death. If Hinton was the real mastermind, he would have found some other dupe, and the discoveries at Piltdown would have continued.

Dawson also tried to pass off other frauds — a plagiarized book as his own, cryptozoological creatures and bogus artefacts. Dawson consistently appears as the most likely perpetrator of the Piltdown hoax. What Gardiner has done is to discover Dawson's accomplice.

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Energy, not bombs

SIR — It is unfortunate that *Nature* (**381**, 267; 1996) has joined in the criticism of India's approach to a Comprehensive Test Ban Treaty (CTBT).

In contrast to that of its neighbour, China, the primary focus of India's nuclear programme has been to provide energy for its people. As a result, India's nuclear energy programme is the second largest indigenous programme in Asia, after that of Japan. Furthermore, India's capability since 1960 to manufacture nuclear weapons is well documented (K. D. Nichols, *The Road to Trinity* 351–352, William Morrow, New York, 1987). India was one of the few countries with this capability (although it faced consistent threats on its borders) to have shown restraint. It was only after the emergence of the alliance between the United States and China in 1973, however, that India felt threatened and demonstrated its capability with a sophisticated nuclear explosion test in 1974. Since then, India has not tested any other device.

Rather than criticizing India, therefore, the declared nuclear powers should set an example and move towards total nuclear disarmament within a realistic time-frame. This would give the CTBT universal appeal. In fact, the late Rajiv Gandhi suggested a time-frame for the total liquidation of weapons of mass destruction. Quite recently, Pope John Paul II expressed similar views.

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History lesson

SIR — I refuse to accept Henry I. Miller's comparison of the Nazis with groups in Germany opposed to field tests (*Nature* **381**, 362; 1996). I also reject the term “Entartete Forschung”. I do not agree with the activities of groups in Germany opposed to genetic engineering, but to equate them with the criminals of Nazi Germany is just stupid.

During my two-year stay in the United States I met too many people who may have learned a lot, even history, but have understood nothing. Miller's letter is another example.

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Jury still out on cold fusion?

SIR — The News story “Scientists lose cold fusion libel case” and part of a leading article in the same issue (*Nature* 380, 369 & 367; 1996) might convince readers that an Italian court has actually found a proof of misconduct by Martin Fleischmann, Stanley Pons and three Italian scientists (two of whom are the present writers).

In fact, the court’s verdict, against which we shall appeal, falls short of your hasty conclusion. The court, following the conclusions of the court-consultant, acknowledges (page 13) that “the cells with electrodes of treated palladium with heavy water solutions, undoubtedly [our italics] produce an unexplainable quantity of heat”, and there is no mention of fraud or misconduct anywhere in the text.

Unfortunately, it is on the widespread innuendo, defamation and vituperation that have characterized the response in scientific circles that the court bases the right of the journalist to report such defamations as “the voice of the scientific community” with no regard to the honour of dedicated and honest individuals. On page 13, the Italian text reads: “One must thus reckon that the severe critiques [a clear understatement!] of the promoters of cold fusion in the articles of Pace Giovanni Maria are justified by the existence of a strong opposition in the scientific community not only to the theoretical bases of the research but also to the way in which experiments were conducted, the data were published and the conclusions on the prospects of the research programme were drawn.”

One might compare such a verdict with the hypothetical verdict of a German court in the 1930s dismissing the libel case of a Jewish group against a journalist of the *Volksischer Beobachter* on the grounds that antisemitism was the prevalent attitude in Germany at the time. Traces of such a doubtful ethics can be found in several leading articles in *Nature* about the “cold fusion affair”. In particular we have in mind the question at the end of David Lindley’s Commentary article (*Nature* 344, 375-376; 1990): “Would a measure of unrestrained mockery, even a little unqualified vituperation, have speeded cold fusion’s demise?”

The problem with the enemies of cold fusion, the True Unbelievers, is that they are a bit lazy. Even Lindley acknowledges that “[a]lthough cold fusion was [note the past tense even in the spring of 1990!], in terms of ‘ordinary’ physics, absurd, it was not obviously so; it contravened no fundamental laws of physics”, but the leaders of the scientific community don’t want to do their homework and prove their point (either experimentally or theoretically). They simply dismiss everything that supports this new scientific development and, when cornered by fact and logic, they

explode in a burst of insults, while, when required to give a proof of their charges (fraud like cold fusion should in the end be proven!), they appeal to the right of “free press”. It is for this reason that we will appeal against this “liberal” verdict and will continue to defend the right to “free research and discovery” in a scientific community where too many ‘ayatollahs’ pretend to know in advance what is right or wrong. This attitude has the obvious risk that such loud editorials, statements, judgements and the like will eventually bring an unbearable mass of ridicule upon their authors. And, judging from the positive evidence on cold fusion being accumulated, it is very likely that this misfortune will happen very soon to the most vocal among them.

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The g factor

SIR — N. J. Mackintosh in his review of *The g Factor* by Christopher Brand¹, is incorrect in stating that neither Brand nor anyone else “ever established that inspection time [IT] owes its correlation with IQ scores to its correlation with g”.

IT is the speed with which a person can make a simple visual (or auditory) discrimination. The g factor is the largest or highest-order factor common to all of the tests in a battery of psychometric measures of cognitive abilities, such as the collection of diverse subtests typically used to obtain an IQ score². A factor analysis of the correlations among various cognitive tests invariably yields a general factor (g) and two or more other factors that are correlated with more specific abilities, such as verbal, spatial and memory. A meta-analysis of all the reported correlations between IT and various IQ and other psychometric tests published before 1989 gave an average correlation of -0.54 after correction for artefactual sources of error (-0.30 before correction)³.

The question raised by Mackintosh, put more precisely, is whether IT is correlated more with the g factor than it is with factors other than g in test batteries used to measure IQ. There are three ways to answer this question empirically: (1) factor analyse IT among a battery of psychometric tests, (2) correlate g factor scores and non-g factor scores derived from the various sub-

tests of an IQ battery, and (3) correlate the column vector of (a) the correlations between IT and each of the subtests, and (b) the column vector of the correlations between each of the subtests and g. Application of these methods shows that the g factor is in fact the main source of the commonality between IT and IQ.

IT had larger correlations with g than with any other psychometric factors (independent of g) in two studies^{4,5}. Both studies also show a high correlation between the vectors of IT and g subtests. In a factor analysis of the correlations between IT and eleven psychometric tests (Raven’s Advanced Progressive Matrices and the ten subtests of the Multidimensional Aptitude Battery) administered to 101 college students, IT has its largest correlation with the g factor, a much smaller correlation with the spatial factor and a near-zero correlation with the verbal factor, both factors independent of g. (The correlations used for this factor analysis, all from one study, can be found in refs. 6 and 7.)

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Last of the...

SIR — Mark Smith (*Nature* 381, 272; 1996) is of course quite correct in pointing out that the last extant representative of any particular lineage may be called a relict, but unfortunately this word has the wrong connotations: it evokes more of past greatness than present catastrophe and it is more suggestive of persistence than extinction. As for the other words proposed — Chaucer notwithstanding — ‘ending’ is a less ugly word than ‘ender’, but it does sound somewhat pathetic, as Elaine Andrews pointed out (*Nature* 381, 272; 1996). Her suggested ‘terminarch’, on the other hand, is certainly stronger, but is such a ‘positive ring’ really appropriate here? For what we hope are obvious reasons, we should like to propose the shamelessly romantic term ‘mohican’; it is more poignant than pathetic and alludes straightforwardly to the tragic.

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Hazards of the passive voice

SIR — Simon Leather (*Nature* 381, 467; 1996) blames every vice from colloquialism to fabrication in scientific writing on the decline in use of the passive voice. He also suggests that “use of the passive voice encourages precision...”.

Perhaps he should also note that only the passive voice can engender a classic grammatical howler: the dependent clause with misattributed subject, as in “by standing at a distance, an unbiased viewpoint is much more likely to be reached”.

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SIR — Leather prefers the passive to the active voice, which he even thinks is partly responsible for the increase in fraud over the past 20 years. I think he is, quite simply, mistaken. He is certainly in a minority. Few authors of books on English usage or scientific style manuals prefer the passive.

Leather believes the passive allows researchers to stand at a distance from their work, but scientists cannot help but become involved in their work. Investigators must also not be allowed to stand back and disclaim responsibility. Leather says that only the passive voice demands correct tenses, but good writing, whether in the active or passive, demands correct tenses. Authors may “show no consistency of use”, but that is not because they write in the active; it is because they can't write well.

Whatever has led to the increase in scientific fraud — if indeed there has been an increase rather than an increased awareness of fraud — is it likely that the active voice has been an important factor? Pressure to publish and pressure to keep one's job are surely likelier causes. And even 40 years ago papers were written in the active voice: “We wish to suggest a structure for the salt of deoxyribose nucleic acid” (J. D. Watson & F. H. C. Crick *Nature* 171, 737–738; 1953).

The heart of the matter is that a good writer knows when to use the active and when to use the passive. When Leather writes that, “as used by many of its adherents, [the active] does no favours to the English language or science”, he is really just repeating what he said in his first paragraph, and what is undeniably true. The current generation of science graduates has been let down badly by their education (an expression better in the passive) and many cannot write clear English, whether in the active or in the passive.

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SIR — Leather correctly points out that the active voice can impart a personal agency

where none were better shown. But so too the passive voice can hide an agent inappropriately, as in the ever-so-blameless “mistakes were made”. Active and passive voices differ not only in whether they name the agent but also in where they position parts of a sentence. Thus, in an essay about “great discoveries”, one writes “Great discoveries are published by *Nature*”. But in an essay about the journal, that sentence becomes “*Nature* publishes great discoveries”.

Where subjects and objects are put in a sentence determines in part how coherently individual sentences flow together to form a paragraph. Active and passive voices each have important roles in writing. The clear and graceful writer ignores injunctions against the active or passive voice, and instead chooses an appropriate voice for every sentence.

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Is the Pope an alien?

SIR — Amos addressed the interesting question of chance results and their proliferation¹ which was later discussed by others^{2–4}. In this context, we want to draw attention to a more fundamental problem of statistical inference.

Aristotle investigated situations in which conclusions can be derived from premises. A well known example is that from the two premises (1) all humans are mortal, and (2) Socrates is human, it can be concluded (3) therefore Socrates is mortal. A necessary prerequisite for the validity of this type of syllogistic reasoning is that the premises are absolutely sure. Absolute certainty, however, is not the subject of statistics.

This reasoning becomes invalid when applied to probabilistic premises. If, for example, we randomly pick a human being, the probability that it is the Holy Father is extremely low — it is 1:6 billion = 0.00000000017. Therefore (1) if an individual is human, it is probably not the Pope ($P < 0.00000000017$); (2) John Paul II is the Pope; (3) therefore, he is not a human being ($P < 0.00000000017$). Which is obviously not sensible.

This example proves that the change from absolute certainty to probability makes the syllogistic reasoning false. Unfortunately, this is formally exactly the procedure that is applied in statistical hypothesis testing: (1) if the null hypothesis is true, these data are unlikely ($P < 0.05$); (2) the data have occurred; (3) therefore the null

hypothesis is wrong ($P < 0.05$).

That this type of inference is wrong was noticed by Aristotle more than 2,000 years ago. It has also been sporadically discussed in the literature for several decades⁵. Nevertheless, this fallacy is still in use, probably because no alternatives are available. Does anybody know a way out of this dilemma?

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Barbaric century?

SIR — As a scientist whose life almost spans the twentieth century, I believe I have a right to comment on the letter from John Evans (*Nature* 381, 362; 1996).

He believes that future historians will consider the twentieth century as “quite the most barbaric in all history”. He claims that the two major wars were in part due to scientific ideas and the ready willingness of scientists to devise lethal weapons. He also thinks that scientists seek an undeserved status. Future historians may rate the global eradication of smallpox an ‘A’, the overgrowth of population an ‘F’.

There was less involvement of scientists in the First World War than in the Second. I gave my five best years (34 to 39) to assist the Allies because I thought that three dictatorial regimes were seeking to gain control of vast areas of land and large numbers of people in a very cruel way, by military might. Scientists in the United Kingdom and the United States did have some effect and probably saved many lives. Among the “lethal weapons” that I helped to develop was radar, now found in all commercial ships, aircraft, airports and weather stations.

An important component of science is experiment. I am an experimentalist. I do not see “abstract impersonal logic” in the observation that a mould generates an antibacterial agent. Nor that the track of a particle in a magnetic field curves oppositely to that of an electron.

I qualify as one of the “molecular biologists who go to church”. I do so for my own reasons. Among those reasons is no desire to deny or modify the knowledge gained by the careful study of nature. Together with Lord Rayleigh I consider this knowledge to be revelation of the works of a supreme being. I pay them respect and in doing so pay respect to the supreme being. I consider this to be wise.

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EPA science: casualty of election politics

David L. Lewis

The struggle between the Congress and the White House over environmental issues provides an opportunity for improving science at the US Environmental Protection Agency, a chance that may be lost in the fray of election-year politics.

JUST over a quarter of a century ago, when I began working at the US Environmental Protection Agency (EPA)'s research laboratory in Athens, Georgia, our scientists devoted most of their time to hands-on research aimed at solving the country's environmental problems. The agency's technical workforce consisted mainly of federal scientists, including researchers and technicians, along with stay-in-school students from nearby universities. The EPA now has about a third fewer scientists, and their time is largely consumed by administrative red tape, overseeing contracts and other funding agreements designed to accommodate increasing workloads and a diminishing federal staff.

Citing Superfund and other programmes that have used an increasingly small fraction of their resources to address environmental problems, Newt Gingrich (Republican, Georgia), Speaker of the House of Representatives, has called for a new era of environmentalism, one that is "scientifically sound, economically rational and politically popular"¹. So far, however, all congressional efforts to achieve this new era have been widely perceived as wholly anti-environmental.

Organizational gridlock

Various independent studies have assessed the status of the EPA's Office of Research and Development (ORD), which is responsible for providing the scientific basis for regulatory activities^{2,3}. Collectively, these present a unified picture of an organization plagued with problems typical of large government bureaucracies. The studies recommend putting into place various measures, which have been reinforced by the National Performance Review (NPR)⁴, including downsizing and decentralizing the top-heavy Washington management structure, reducing the overall ratio of supervisors to workers, giving more power to lower management levels and eliminating redundant programmes and excessive red tape.

Whereas the problems encumbering the agency's scientists have accrued over almost three decades, a major shift in resources from in-house to extramural research during the past ten years is largely responsible for science at the EPA reaching a state of crisis. ORD policy directives and budget figures track the course of this transition (Fig. 1), which arose because Con-

gress increased the EPA's funding levels and workloads while the executive branch reduced the size of the federal workforce. Over a two-year period beginning in 1992, ORD issued about 200 directives on how to manage extramural resources using funding mechanisms that do not lend themselves well to the conduct of research. Many of these later became permanently embodied in ORD's manual on policies and procedures.

By late 1993, the organization charged with ensuring that US environmental regu-

logical phenomena remain by far the weakest links in environmental protection. Relative to our understanding of biological processes, we know much more about how fast pollutants react chemically in the environment, to what degree they are bound by sorption and to what extent they are transported by diffusion, volatilization and other physical processes. Understanding how most living organisms interact with chemical and physical changes in their environment, especially at complex ecological levels, is a particularly daunting challenge. Yet, without this understanding, our knowledge of chemical and physical aspects of environmental science yields only the framework of a vehicle in which we can put the engine one day, once it is built.

Historically, the number of EPA professionals educated in the life sciences has been low compared with those trained in the physical sciences, and has been decreasing in recent years (Fig. 2). Reflecting this disparity, only a third of the internal grants awarded so far by ORD support projects in the biological sciences.

Most organic chemicals undergo structural changes mediated by organisms living in soil and water, often very rapidly. Without understanding these processes, how can the EPA reliably determine whether chemicals should be permitted for manufacture under its Pre-Manufacturing Notification process, if wastes should be deemed hazardous under its Hazardous Waste Disposal Rule or how they should be remediated under its Remediation Feasibility Implementation Study? To look at this from another perspective, consider what it would be like for the Centers for Disease Control and Prevention to use this approach. Begin by erasing most of their knowledge about the biological aetiology of human disease, then give them independent regulatory authority. Let them take their best guesses at, for example, what steps should be taken to prevent outbreaks of influenza and other infectious diseases. Finally, codify their guesswork and conservative safety factors into costly regulations for which noncompliance carries fines and imprisonment. Few people would support such an irrational approach to protecting public health; yet this is basically what happens when environmental regulations are promulgated and enforced without knowledge of how most living organisms interact with environmental changes.

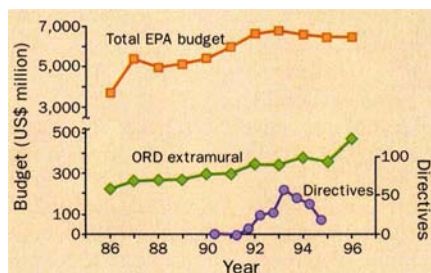


FIG. 1 The EPA's Office of Research and Development (ORD) budgets for contracts, cooperative agreements and other extramural support mechanisms, numbers of directives issued by ORD for guidance in managing extramural support, and total EPA budgets. (Source: US EPA.)

lations are scientifically sound became hopelessly gridlocked⁵. With scientists largely untrained to manage extramural funding mechanisms and deal with a quagmire of government rules, regulations and policies, they and their front-line managers became targets of investigations by the EPA's inspector general. Allegations of mismanagement abounded as the director of the EPA's research laboratory in Duluth, Minnesota (the first of several laboratories to be investigated), was fired and other managers and scientists were either dismissed or sent on leave without pay. They were, however, reinstated by federal judges, who ruled that the agency's allegations were without merit and that investigations had been carried out in bad faith⁶⁻⁸.

Vehicle without an engine

Because environmental regulations are aimed at minimizing the negative impact of humans on other living organisms, regulatory activities are driven by concerns of a biological nature. Nevertheless, understanding and applying knowledge of bio-

Broad vision, bold steps

The EPA recently realigned its twelve research laboratories, three field stations and four assessment centres along functional lines under a risk-based paradigm, resulting in the establishment of three national laboratories and two centres⁹. The agency also committed itself to developing a strategic plan for science, created an investigator-initiated internal grants programme, increased support for its extramural grants programme fivefold (from \$20 to \$100 million) and established a peer-review process for all scientific and technical products.

Some of these changes, such as formally defining its mission and instituting a more comprehensive peer-review process, will undoubtedly strengthen the agency's science. On the other hand, some changes have a clear potential for diminishing productivity and undermining the quality of the science. By centralizing research into mega-laboratories, the EPA's scientific organization will tend to follow the big-bureaucracy path previously forged by the agency's Washington headquarters and regional offices. In time, this could greatly reduce the fraction of resources spent actually doing research as opposed to managing it. ORD has already assessed that its national laboratories need an additional 22 administrative positions each to get them started. To house them, the EPA has begun by targeting around \$250 million in construction costs for a new building complex in Research Triangle Park, North Carolina.

Administrative duties, which previously allowed scientists to spend only half their time doing research³, have become more onerous, with managers and scientists responding to requests from national laboratory headquarters as well as from Washington. Ecologists are concerned that at a time when they should be putting more scientists and resources into research laboratories dispersed in the field, centralization of the organization into national laboratories will damage their basic ecological research efforts. And although it is true that ORD should devote a greater portion of its resources to funding extramural research, recent increases in this category are part of a plan exclusively to use grants instead of cooperative agreements, the only extramural funding mechanism that allows agency scientists to work hand-in-hand with researchers at universities and other research institutions. Already severely restricted from interacting with their outside counterparts because of agency-imposed travel limitations, EPA scientists and engineers are now more professionally isolated than ever before.

If EPA is to retain its role as the federal agency responsible for environmental protection, it must develop a broad vision of the science needed and take bold steps to acquire and apply it. This will require more than just improving ORD. It means mak-

ing fundamental changes in the structure and mission of the agency as a whole so that science leads rather than trying to catch up with the promulgation of environmental regulations. Clearly, we already have an adequate understanding of the effects of many of our activities that directly affect the environment. So minimum-level national environmental standards for basic environmental protec-

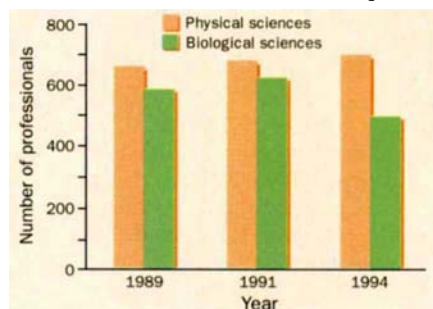


FIG. 2 Number of professionals in the EPA's Office of Research and Development (ORD) trained in mathematics, engineering, chemistry or physics compared with biological sciences. (Source: ORD, *Workforce 89, 91, 94*.)

tion can already be set in many areas with reasonable confidence. Issues where the effects of our activities on living organisms are largely in question from a scientific standpoint are a different matter. These need recommendations, not regulations.

In many cases, protecting public health through recommendations made by a non-regulatory agency (the Centers for Disease Control and Prevention) has worked well and provides a reasonable model for environmental protection. To say the least, it is odd that the federal government protects public health through voluntary recommendations where the underlying science is strong, and the environment through mandatory regulations where the scientific basis is weak. This does not mean that we should wait until the most minuscule uncertainties are resolved in environmental issues, only that the science should be both compelling and widely accepted before federal regulations are promulgated.

The philosophy of federally mandated environmental protection has been to err on the safe side by making it difficult and expensive to do anything that changes our natural environment. This approach is based on the assumption that every change wrought by mankind ultimately has a net negative impact on the long-term health of the environment. Until we have a better understanding of how living organisms interact with the environmental changes brought about by our activities, especially the more subtle ones, we cannot know whether precluding any of these changes will ultimately be detrimental or beneficial.

To provide the overall organizational structure for establishing scientifically sound environmental policies, a cabinet-

level Department of Environment should be created, pulling together the EPA with parts of other agencies and departments involved in the environmental sciences, such as the Departments of Energy, Agriculture and Interior, and the National Aeronautics and Space Administration. In particular, many of the Department of Agriculture's field stations due to be closed in areas where farming is no longer important could be transformed into environmental research laboratories under the direction of the EPA's regionally dispersed ecological research divisions. These could interact with communities on local environmental issues across the country now that environmental concerns are as ubiquitous as agriculture once was, and serve as sentinels of developing environmental trends. Finally, a leading environmental scientist should be appointed as head of the EPA to oversee major changes aimed at achieving a solid scientific basis for federal environmental rules and regulations.

Congress has placed on the national agenda the need to rectify the EPA's inadequate basis for supporting its regulatory process with sound science. It is an unprecedented opportunity to improve an agency that, like all federal bureaucracies, resists change. Environmentalists, on the other hand, have effectively portrayed efforts to alter environmental regulations and the agency that enforces them as a prescription for environmental destruction. But any benefits to the environment today will quickly vaporize if the end result of these battles is that we lose the opportunity to make bold changes, which are needed for building a scientifically sound foundation for environmental protection. □

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This commentary represents the author's personal views, and not those of the US EPA.

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Kings, tree rings and the Old World

Colin Renfrew

Chronologies of the earliest history of the Near East have depended on interpretation of lists of Egyptian kings and such. But the best prospect for an absolute timescale lies in dating wood from Anatolia (modern-day Turkey).

THE goal of constructing a precise, reliable chronology for the archaeology of the eastern Mediterranean, based on tree-ring dating and supported by radiocarbon determinations, is now clearly in sight. It is Anatolia that has yielded the necessary samples of ancient wood. So when the Anatolian tree-ring sequence can confidently be used to give absolute dates for the time range from 3000 BC, the entire prehistoric and early historic chronology of Egypt and the Near East will have to be revised, using the more secure chronological framework that dendrochronology can offer.

On page 780 of this issue¹, a team from Cornell, Heidelberg and Reading present the latest work towards anchoring the 1,503-year floating dendrochronology produced for Anatolian wood samples over a time span covering the whole of the second millennium BC, and several further centuries both before and after. Kuniholm *et al.*¹ use radiocarbon-based 'wigggle-matching' to try to fix the absolute dates for this floating sequence more precisely. (Wigggle-matching involves matching specific irregularities on the master tree-ring calibration curve with irregularities in a series of known-interval radiocarbon dates from a given archaeological wood sample.) A second step in their argument is to equate an exceptional growth event recorded in trees at Porsuk in south-central Anatolia with a widely recognized special marker event seen at 1628 BC in the already well-established tree-ring chronologies for the Northern Hemisphere. If correct, this would indeed anchor their Anatolian chronology to within a single year, with wide-ranging archaeological consequences. In the last section of their article, they revive the suggestion that the cause of this special marker event

was the volcanic eruption of the island of Thera, near Crete, during the Aegean late Bronze Age², thereby rekindling an already heated archaeological controversy³.

There is nothing more tantalizing than a 'floating' tree-ring chronology. Secure overlaps may be obtained from long-lived wood samples from a number of sites, so that relative ages are securely established, with a precision of just a year or so. But to 'anchor' a floating chronology securely, a continuous sequence of samples spanning the entire period ranging back from the present to the time in question is ideally required. This task, which is indeed the long-term objective of the Cornell Dendrochronology Laboratory, will one day be accomplished. But meanwhile, the much less precise technique of radiocarbon dating must be invoked in place of a secure and precise absolute dating. It can be made more precise by using it as a link, through the technique of wigggle-matching⁴, to existing long tree-ring sequences, which are now available in Europe (both from Irish^{5,6} and Central European oak⁷) and in the United States (bristlecone pine^{8,9}).

Kuniholm and his colleagues have been remarkably successful in obtaining wood and charcoal samples from a wide range of archaeological contexts in Anatolia and the Aegean⁸. Their absolute tree-ring sequence now runs back from the present to AD 362, allowing the precise dating of a whole range of early Byzantine buildings. In addition, they have further floating sequences before the 1,503-year Bronze and Iron Age sequence discussed in this issue. Among these is a 570-year floating chronology for the early Neolithic site of Çatalhöyük (dated from about 7000 BC to about 6500 BC by radiocarbon determination) and a 300-year floating sequence from the important Turkish site of Arslantepe, which has

strong links with the Uruk culture of Mesopotamia around 3000 BC. One day, these floating chronologies will be integrated into, and form part of, a master absolute chronology for Anatolia and the Near East, on which the remainder of the absolute dating for much of Old World later prehistory and early history will come to rest.

Already, many scholars will be content to use the arguments presented here by Kuniholm *et al.* as the basis for an absolute chronology which, following their wigggle-matching and their long-range special marker link for 1628 BC, can currently be given a beginning in 2220 BC and an end in 718 BC (until the chronology can be lengthened by further relevant tree-ring samples from suitable archaeological deposits). In the first place, their chronology naturally dates those Anatolian sites from which their samples come, most notably the Old Assyrian trading station at Kültepe for the earlier part of the time range, and the great burial mounds at Gordion for the later part of the range (see photograph). Hitherto, the *karum*⁹ (trading centre) at Kültepe has been dated by the documents there, written on clay tablets, which allow synchronisms to be established with the historically based Old Assyrian chronology of the Near East. But the stage is now being reached at which the greater precision, and perhaps the greater reliability, of the Anatolian tree-ring chronology will lead to a reversal of the procedures of dating.

Hitherto, it has been the historical chronologies of Egypt and the Near East, laboriously worked out by generations of scholars through careful interpretations of the Egyptian and Near Eastern king-lists and annals (with all their various problems of historical exegesis), which have offered the bedrock for the absolute chronology of



P. I. Kuniholm

The Midas Mound tumulus at Gordion, which lies about 100 km southwest of Ankara in Turkey and was constructed around 718 BC. The mound is made-made and massive (53 m high), and contains a burial chamber for a wealthy man — hence the Midas connection. Tree-ring data from timbers inside the mound provided the foundation of the chronology of Kuniholm and colleagues¹, which runs from 2220 to 718 BC.

the eastern Mediterranean^{10,11}. Areas such as Anatolia, which could be considered peripheral, and which certainly lacked sufficient written records to offer an independent chronological system, were inevitably dated on the basis of the links which could be established with those well-dated areas of Egypt and the Near East.

All this is now on the brink of being reversed. As soon as we are entirely confident that the Anatolian absolute chronology is soundly based, it will offer a more secure and a more precise chronological route. Anatolia will be the chronological 'known', and the historical chronologies of Egypt and the Near East will assume a secondary status, being calibrated according to the links which they can establish with what will in future be the primary area for the master chronology.

But has that day yet been reached? The wiggle-matching, based on 18 radiocarbon determinations made in the Heidelberg laboratory on samples from a single juniper log from the Midas Mound tumulus at Gordion, looks sound enough, but not as precise as one might wish. Kuniholm *et al.* claim that the chronology is anchored by the "remarkable growth anomaly", seen in the 36 trees from Porsuk in south-central Anatolia, which they choose to equate with the major growth anomalies at 1628/1627 BC in Europe and the United States. But before re-structuring the entire chronology of the Near East on the basis of the new primacy of this Anatolian chronological system, we must ask just how compelling is this equation between the Porsuk growth event and the widespread event of 1628 BC?

The generally accepted explanation for the 1628 BC event is that it was caused by a major volcanic eruption, providing a dust mantle which drastically reduced solar radiation reaching the Earth's surface, thus generally impairing tree growth, and leading to the frost damage seen in 1627 BC in the Californian bristlecone pines¹². What Kuniholm *et al.* have not sufficiently explained is how this dust mantle will have produced the "remarkable growth anomaly", where the individual juniper trees put on annual rings greater in width than normal growth by a factor between 3 and 8. They argue that this came about through "unusually high and sustained soil moisture content and a sharp reduction in mid-summer evapotranspiration". Such may have been the case, but a fuller explanation of the Porsuk growth event is clearly needed before it can be accepted as the product of a reduction of received solar radiation.

Kuniholm *et al.* complicate the story further by reasserting the long-standing suggestion¹² that the special Northern Hemisphere marker event of 1628 BC was itself caused by the eruption of Thera, conventionally dated more than a century later than this. The alternative cause might be an as yet unidentified volcano, whether in Iceland or Alaska or elsewhere in the

Northern Hemisphere. Recently the advocates of the conventional (lower) chronology have taken great comfort from the discoveries of pumice¹³, presumably deriving from the great eruption at Thera, in strata which follow those of the late Hyksos palace recently unearthed at Tell Dab'a in Lower Egypt. Deposits associated with that palace contained fragments of fresco paintings of Minoan character closely resembling some of those found in Thera and dating from the period there immediately before the great eruption¹⁴.

Because these pumice finds seem to link the Thera eruption securely with a time at the beginning of the XVIIIth dynasty of Egypt (usually¹⁵ set at about 1550 BC), to adopt the date of 1628 BC proposed by Kuniholm *et al.* would imply very substantial changes to the historical chronology of Ancient Egypt. That cannot be ruled out, but such changes would need to be based upon more than a suppositional correlation between the Thera eruption and the 1628 BC event seen in the Northern Hemisphere tree rings and ice

cores (although the ice cores were initially interpreted¹⁶ as indicating a global event at 1645 BC). One grain of Thera tephra at the appropriate point in a single Greenland ice core would be enough to establish a sound link going beyond mere supposition. Alternatively, an unassailable causal link between the Thera eruption and the growth anomaly in the Porsuk trees would do very nicely.

Until we have these necessary linking data, there is too much supposition in the arguments for one to feel that all doubt has been banished. One day we shall have such data, and very probably it will be provided by Kuniholm and his colleagues in the Cornell laboratory. Their work offers the best hope we have for a really sound chronology for the later prehistory and history of the Near East and Egypt, and indeed the eastern Mediterranean in general. But their work is not yet complete. □

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SPONGIFORM ENCEPHALOPATHIES

Between cows and monkeys

Adriano Aguzzi

WHAT would constitute definitive evidence that bovine spongiform encephalopathy (BSE) has spread to humans? This question — the importance of which cannot be overstated — is exercising scientists as much as politicians and public-health officials, and for several months it has been a front-page issue in the European press. The latest storm was provoked by reports in April of a new form of spongiform encephalopathy occurring in young people in Britain¹ and, at least in one case, in France². The salient feature of this disease (which was termed, perhaps somewhat conservatively, 'variant of Creutzfeldt-Jakob disease' or vCJD) is the presence of abundant 'florid plaques', large cortical deposits of pathological prion protein (PrP^{RES}) decorated by vacuolated brain tissue in a daisy-like fashion (*a* in the figure).

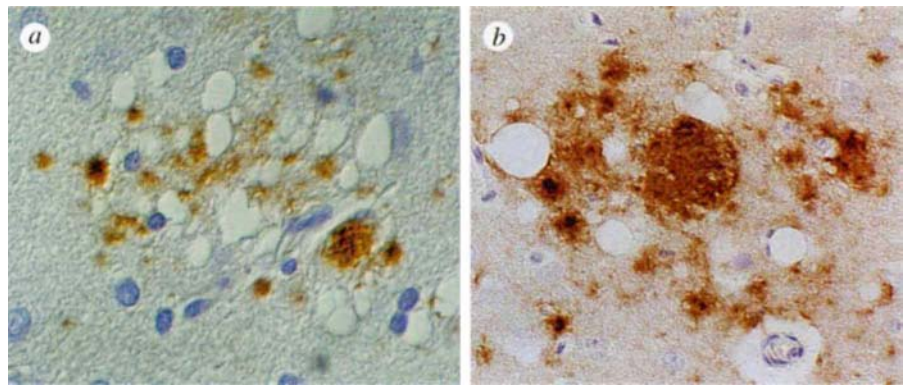
Two weeks ago came news of a study with macaque monkeys, released at a press conference, which reinforces the suspicion of a link between BSE and vCJD, and adds pathogenetic evidence to epidemiological data. Elements of the study concerned, which was carried out by Lasmézas and colleagues, now appear in *Scientific Correspondence* on page 743 of this issue³. The authors have found that intracerebral inoculation of three macaques with brain extracts from cattle suffering from BSE produced a brain disease with plaques identical to those of vCJD patients (*b* in the figure), whereas no plaques were seen in two macaques inoculated with sporadic human CJD. The monkeys are reported to have developed a syndrome consisting mainly of prominent cerebellar damage, but also more exotic symptoms such as "voracious

appetite". Gajdusek observed this latter characteristic in Papuan patients afflicted with kuru—a prion spongiform encephalopathy propagated through cannibalistic rituals—and it has been ascribed to hypothalamic damage.

The BSE agent shows a characteristic behaviour which has never been seen before in transmissible spongiform encephalopathies. For example, it is highly promiscuous in its choice of hosts. Unlike its counterpart in sheep, mice and hamsters, it appears to infect animals of other species easily, especially when transmitted orally. The molecular basis for these 'strain-specific' traits is completely unknown. Because kuru also exhibits abundant plaques, one might argue that the presumptive oral route of application is the main determinant of neuropathology in vCJD. The observations of Lasmézas *et al.* run counter to this argument and suggest that the neuropathological findings characteristic of vCJD in humans represent a specific trait of the BSE strain of prions in primates. (Here I will use 'prion' as a generic term to refer to the infectious agent involved in transmissible encephalopathies, irrespective of its true physical nature. There is strong, but as yet not clinching, evidence that the agent is an aberrant isoform of a normal cellular protein called PrP^C. The agent is thought to recruit PrP^C and transform it into further infectious protein.)

The results of Lasmézas *et al.* of course leave many questions, the first of which is whether the macaque system is a realistic model of the situation in humans. Although the inoculum used is large by molecular criteria, it is equivalent to only 50–100 mg of infectious brain homogenate. It is unsettling that these amounts are well within the range of brain tissue present in commercial food products for human consumption until a few years ago. On the other hand, however spectacular (and worrying) these findings are, it should be stressed that transmission was by direct intracerebral inoculation, and we can hope that the oral route of administration will be considerably less efficient. Although there are no available data on the relative efficiency of oral as opposed to intracerebral transmission of BSE to primates, we know that as little as 500 mg of brain extract administered orally produced encephalopathy in one out of six sheep¹.

Next, how can we go about estimating the risk of contracting the disease after exposure to the BSE agent? One issue of utmost interest is the possibility of genetic susceptibility to infection. By extrapolation of the findings from transgenic and knock-out mice, there are probably two key factors: first, the primary sequence of the *PRNP* gene which encodes the normal cellular prion protein (PrP^C); and, second, the availability of PrP^C for conversion into pathological PrP^{Sc} by the infectious agent



The similarity between the neuropathology of the new variant of CJD (vCJD) and that seen by Lasmézas and colleagues³ in the macaques three years after intracerebral inoculation with brain extracts from BSE-infected cattle. *a*, Brown immunohistochemical stain shows the prion protein in the 'florid plaques' characteristic of vCJD in the brain of a 26-year-old French patient (in a cortical biopsy taken several weeks before death). *b*, Plaques evident in the macaques' brains.

(which may or may not be identical to PrP^{Sc}). As far as the first issue goes, the polymorphism at codon 129 of the *PRNP* gene (which encodes either methionine or valine) is emerging as a crucial parameter⁵. All the British vCJD patients, as well as the case from France, were homozygous for Met 129, while a further suspected patient is homozygous for Val 129 (J. Collinge, personal communication). Therefore, Met/Val 129 heterozygosity (prevalence of 51 per cent in Caucasians) would appear to be a protective factor. Within the framework of the protein-only prion hypothesis⁶, this finding may indicate that heterozygous individuals produce two PrP^C moieties which are subtly different from each other, and reduce the efficiency of PrP^C-to-PrP^{Sc} conversion by reciprocal competition. Regrettably, in the macaque experiments this issue has not yet been studied in detail: the status of codon 129 in the three inoculated animals was not determined, and indeed it is unclear whether codon 129 is polymorphic in the macaque.

A further potential risk factor may be represented by the amount of PrP^C present in the brain. It has been demonstrated repeatedly that prion replication relies on the presence of PrP^C (ref. 7), and that mice that are heterozygous for the prion gene (*Pmp*^{+/0}), and have reduced levels of PrP^C, develop a milder form of scrapie after challenge with infectious prions^{8,9}. Moreover, PrP^C is an essential mediator of pathogenesis because chronic exposure of *Pmp*^{+/0} mice to large doses of prions does not induce any brain damage¹⁰. So it will be important to confirm that PrP^C expression levels in macaque monkeys are similar to those of humans.

Another matter to be resolved relates to the mechanisms of neuroinvasiveness. How do prions, when they are ingested orally and travel into the gastrointestinal tract, then move across the body and colonize the nervous system? Several lines of evidence point to lymphatic organs as a

possible link. As shown by Lasmézas and colleagues in another paper¹¹, the neuroinvasiveness of intraperitoneally administered prions is impaired in SCID mice (which suffer from 'severe combined immune deficiency', and have major lymphatic defects), and can be reconstituted by transfer of spleen cells. Although transgenic mouse models are proving helpful in dissecting this problem, they may fall short of reproducing all the peculiarities of the BSE agent. The reason that caution is necessary is that, although BSE prions may replicate in the lymphoreticular system of most species (including sheep, hamster and mouse), they show a surprising reluctance to infect spleen and lymph nodes of cattle.

One obvious question, therefore, which needs to be addressed urgently, is whether BSE prions cause vCJD in macaques even when administered orally. If so, what is the minimal infectious dose? Such studies are demanding because they require exposure of a large number of primates to serial dilutions of the infectious agent over many years. Further experiments will also have to address the molecular basis of the strain characteristics of the BSE and the human vCJD agents. Indeed, a thorough understanding of vCJD strain variation is likely to be a precondition to devising rational approaches to prevention and, perhaps, to treatment. □

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Sound, light and the vacuum

Peter Knight

SONOLUMINESCENCE is a strange phenomenon in which light is emitted when bubbles in liquids collapse. It was thought that the light came from a tiny region of plasma, created and heated by the collapse, but a paper that appeared in *Physical Review Letters* in May¹ proposes a more exotic mechanism, and has attracted an extraordinary amount of attention.

Bubble collapse has been known since the beginning of this century to lead to propeller damage on ships, indicating the dramatically strong forces produced. The fact that the collapsing bubbles emit light

is certainly high enough to allow such temperatures, and everything seen so far is consistent with this high-temperature model. But here is the central puzzle: is the collapsing bubble in sonoluminescence really that hot? The evidence so far is really rather indirect, deriving from the observed tail of the thermal-like spectrum. But if the collapsing bubble is so hot, we would expect to see traces of the existence of high-energy photons — ionization and fragmentation of the liquid, for example — traces that are so far lacking.

Other explanations for the light emission, involving bremsstrahlung, collision-broadened molecular lines and so on, have been proposed. The paper¹ by Claudia Eberlein puts forward a radically different interpretation: that the sonoluminescence is a vacuum radiation phenomenon related to the Casimir effect, converting vacuum fluctuations into real radiation through the rapid acceleration of the dielectric boundary of the bubble. Rather than a collection of individual atoms, the dielectric liquid may be regarded as a continuous

medium with a polarization that responds to electric fields, so the photons are radiated as a consequence of the interaction of the dielectric with the quantized electromagnetic field.

Calculations made many years ago by Casimir to explain long-range intermolecular forces demonstrated the reality of vacuum fluctuations and the zero-point energy. So the idea of vacuum radiation is one with which physicists are perfectly comfortable. Indeed, theoretical work by Hawking and Unruh using modern quantum field theory predicts unambiguously that quantum vacuum radiation exists: photons may be created in pairs parametrically by time-varying situations such as moving mirrors or black-hole boundaries, or anything else that couples pairs of radiative modes together. 'Parametric' here means that some governing parameter is made time-dependent; in this case, it is the boundary of the bubble.

Photon generation by moving boundaries is normally a very small effect, but it is predicted by conventional theory, and one needs neither uniformly accelerated mirrors nor black holes for the effect to exist. The radiation emitted by such a process is thermal-like, in that it possesses a broad-band, featureless spectrum simi-

lar to that emitted by a black body, but no thermal processes need be invoked. This has been known for some years in conventional nonlinear optics, where photons have been created in pairs as two-mode 'squeezed' states of light, where the source medium is made time-dependent by an external laser pump field³.

That work showed that although the state of the combined photon pair is highly correlated, with each photon in the pair born at exactly the same time, individual photon modes looked at in isolation have precisely the excess noise characteristic of thermal light, with an effective temperature governed by the degree of correlations between the pair. However, the light is certainly not thermal; it merely mimics planckian photon statistics. Any parametric process in which correlated pairs are created will have something of this property.

In recent years, many physicists have contemplated the generation of vacuum radiation from moving boundaries. Eli Yablonovich suggested⁴ that the generation of pair photon states by the Unruh mechanism could be seen experimentally from a suddenly moving plasma interface created by pulsed laser excitation of a semiconductor surface. Unfortunately, this does not seem practicable, because the strong plasma radiation will totally mask the weak quantum vacuum radiation. Researchers are currently considering measuring the effect in microwave cavities with very high quality factors and with oscillating walls. And this brings us back to sonoluminescence: there is a chance that the collapsing gas bubble in sonoluminescence naturally provides such a high-quality cavity, because the minimum bubble size is about 500 nanometres. The presence of a cavity of that size makes the emission of visible light very much stronger owing to resonant enhancement.

The new theory is particularly attractive because it does not require the photons to emerge from relatively slow atomic transitions, but uses ideas derived from nonlinear optics, where parametric processes can be very fast. It is known that a sonoluminescence pulse containing a million photons can be generated in a thousandth of the time it normally takes an atom to produce a single photon (around ten nanoseconds for the visible hydrogen spectrum), and some of the conventional theories have difficulties with this timescale.

There is no proof, however, that sonoluminescence actually is quantum vacuum radiation; what Eberlein has shown is that it could be. To prove it conclusively would

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Typical sonoluminescence set-up — the measurement of bubble radius is made using laser light scattering.

was discovered in 1934 by Frenzel and Schultes in Cologne. In 1990, Gaitan and Crum showed how a single bubble in water could be trapped stably and made to expand and contract periodically (typically at about 25 kHz) and to emit light in time with the sound pulses driving the bubble (see figure). But the big surprise came with the discovery in 1991, by the group in Los Angeles headed by Seth Putterman, that each light pulse in this controlled sonoluminescence lasts less than 50 picoseconds!

The spectrum of the emitted light was seen to have a low-frequency tail reminiscent of the Planck distribution characteristic of thermal light. It was assumed that most of the light at higher frequencies was absorbed by the liquid surrounding the bubble, so that all that escaped to be measured was the low-frequency tail of a much wider spectrum. If this radiation is thermal in character, then the material of the collapsing bubble must get extremely hot to emit visible light. Indeed, the temperatures would have to be many tens of thousands of degrees, close enough to the temperatures of thermonuclear plasmas to excite the inertial fusion community².

Careful calculations show that the energy density in these collapsing bubbles

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2. Pool, R. *Science* **266**, 1804 (1994).

3. Barnett, S. M. & Knight, P. L. *J. opt. Soc. Am.* **B2**, 467 (1985).

4. Yablonovich, E. *Phys. Rev. Lett.* **62**, 1742 (1989).

require a demonstration that the photon pairs emerging from the bubble have the non-classical statistics expected from such a correlated origin. This is possible, if very difficult, with present-day experimental techniques.

The vacuum radiation theory of sonoluminescence is based only on conventional quantum field theory developed over many years. Nevertheless, it is novel in that it puts known things together in a different and unconventional way. Because

of the timescale considerations, some kind of parametric effect (such as occurs in the conversion inside nonlinear crystals of one photon into two) is likely to be involved, and quantum vacuum radiation is one such effect. The real test will be to look for the correlations such a model predicts, against the energetic photons predicted from the high-temperature model. □

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TASTE TRANSDUCTION

A bitter-sweet beginning

Sue C. Kinnamon

GUSTDUCIN, a signalling protein found on the inside face of the membrane of taste-receptor cells, was first cloned more than four years ago¹, but its role in transmitting taste sensation has until now remained a mystery. On page 796 of this issue², Wong, Gannon and Margolskee report the effects of 'knocking out' gustducin in specially bred mice. Predictably, these mice lose their sensitivity to bitter tastes, but — and this was unexpected — they are barely able to taste sweet compounds either. So it seems that gustducin is a key participant in both bitter- and sweet-sensing pathways.

Taste transduction begins when taste stimuli interact with receptor sites or channels on the apical membranes of taste receptor cells, causing membrane depolarization and release of transmitter from receptor cells onto gustatory afferent neurons³. Although a vast assortment of

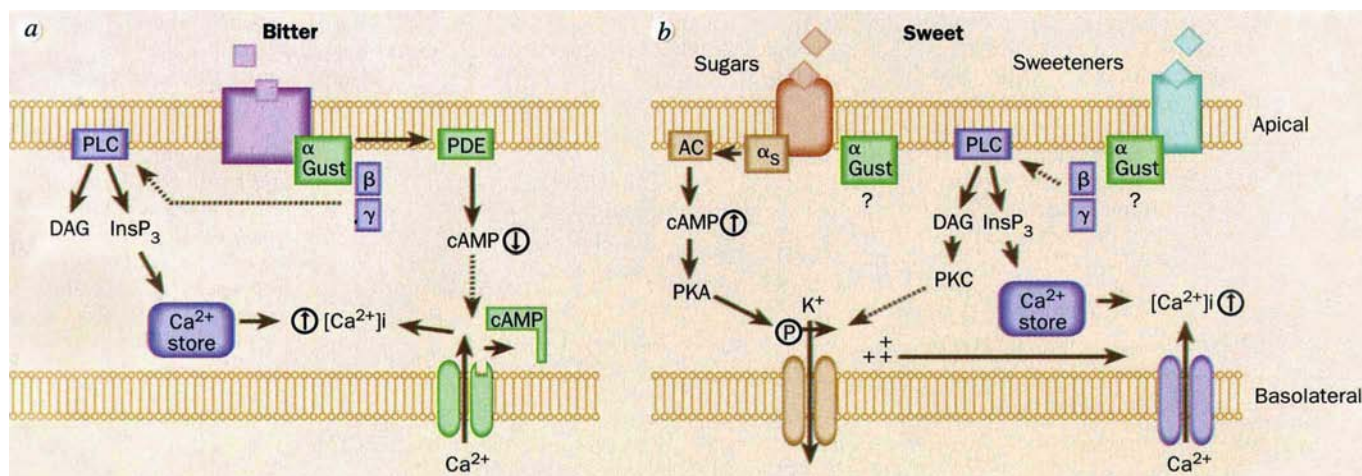
soluble molecules elicit a sensation of taste, humans classify these compounds into a few broad groups: sweet, salty, sour, bitter, and umami, the unique taste elicited by glutamate.

Given the chemical diversity of taste stimuli, it is not surprising that complex mechanisms are required for their transduction. Ionic stimuli such as salts and acids interact directly with apically located ion channels to depolarize taste-receptor cells, but sugars, amino acids and most bitter-tasting compounds bind to specific receptors on the outside of the cell membrane, most of which are coupled to heterotrimeric guanine-nucleotide-binding proteins (G proteins, of which gustducin is an example) and second-messenger systems that relay the signal to the interior of the cell. So far, these receptors have eluded attempts to purify or clone them.

Going for the next component in the signalling pathway after the presumed taste receptors, Margolskee *et al.* looked for taste-cell-specific G proteins. Using the polymerase chain reaction, they cloned the α -subunits of several G proteins expressed at higher levels in taste cells than in surrounding tissues. These include G_i , members of the G_i and G_o families, and the special G protein gustducin, which is expressed exclusively in taste-receptor cells¹. Gustducin is closely related to the transducins, which activate phosphodiesterase (PDE) in the vision phototransduction pathway. Margolskee and colleagues also identified both rod and cone transducin in taste cells⁴, although these were present in much smaller amounts than gustducin. By analogy with the visual system, they considered that both gustducin and transducin should activate PDE in taste cells, leading to a decrease in the cyclic nucleotide (cAMP) second messenger.

Now the Margolskee group has used targeted gene replacement to eliminate the gene encoding the α -subunit of gustducin (a 'null' mutation). Taste deficits were investigated in these null mice using behavioural two-bottle preference tests and by monitoring electrophysiological responses of the chorda tympani nerve to selected taste stimuli. The responses were normal to salty and sour stimuli, as expected, because these stimuli interact directly with ion channels to depolarize taste cells. They were less responsive to two bitter stimuli, denatonium and quinine, and to two sweet stimuli, sucrose and an extremely sweet synthetic sweetener.

The reaction to sweet and bitter stimuli was not completely 'knocked out', but



Possible roles of gustducin in *a*, bitter and *b*, sweet taste transduction. Bitter and sweet tastes are conveyed by different taste-receptor cells⁹. Established pathways are indicated by solid lines and hypothetical ones by dashed lines. *a*, In bitter-sensitive cells, bitter stimuli bind to G-protein-coupled receptors to activate phosphodiesterase (PDE), resulting in decreased levels of cAMP. This may alter the phosphorylation state of intracellular proteins such as ion channels and enzymes, or it may relieve the inhibition of a recently discovered cAMP-blocked cation channel, resulting in Ca^{2+} influx. In addition, bitter stimuli activate phospholipase C (PLC), causing release of Ca^{2+} from intracellular stores. Gustducin may be involved in both pathways: α -gustducin activates PDE, whereas the $\beta\gamma$

subunits may activate PLC. *b*, In sweet-sensitive cells, sugars stimulate cAMP formation, probably through another G-protein α -subunit (α_s) that specifically works on adenylyl cyclase (AC) to make cAMP, which activates protein kinase A (PKA), leading to phosphorylation and closure of basolateral K^+ channels. Synthetic sweeteners act on the sweet-sensitive cells as well, but unlike sugars, they stimulate PLC to produce $InsP_3$ and diacylglycerol (DAG). $InsP_3$ causes release of Ca^{2+} from intracellular stores, while DAG stimulates protein kinase C (PKC), which may phosphorylate the same basolateral K^+ channels. The role of gustducin in sweet transduction is less clear, but could involve regulation of cAMP levels through α -gustducin and stimulation of PLC by $\beta\gamma$ subunits.

only diminished with respect to control mice — the lingering sensitivity could have been due to the presence of transducin, which may rescue the response, but double-gene knockouts would settle that point. It would be interesting to know how Margolske's null mice respond to glutamate, especially as new findings suggest that glutamate may be transduced by a metabotropic glutamate receptor that causes a decrease in cAMP⁵.

The link of gustducin with bitter taste came as no surprise, as it had been known for many years that bitter compounds activate PDE in taste tissue⁶. Consistent with this, Margolske *et al.* have shown that both transducin and gustducin activate PDE from taste cells and that in the presence of taste membranes, the bitter compound denatonium activates transducin or gustducin⁴, suggesting that taste-cell membranes contain a receptor for denatonium which couples to transducin or gustducin.

Some important problems remain, however. Previous studies of the transduction of denatonium showed that denatonium activated the enzyme phospholipase C (PLC) in the cell membrane, leading to the production of a different second messenger, inositol trisphosphate (InsP₃), and release of Ca²⁺ from intracellular stores⁷. As G_i signals through InsP₃, the denatonium receptor might be coupled to a G_q-type protein rather than to gustducin or transducin. Alternatively, the α -subunit of gustducin may activate PDE, while the $\beta\gamma$ subunits activate PLC (a in the figure), as in other systems⁸.

The big surprise is the apparent involvement of gustducin in sweet transduction, because previous studies had shown that sugars increase, rather than decrease, cAMP levels in taste tissue⁹. The prevailing view is that sugars activate adenylyl cyclase, which generates cAMP and results in closure of basolateral K⁺ channels^{10,11} (b in the figure). Synthetic sweeteners stimulate PLC rather than adenylyl cyclase in order to depolarize taste cells⁹, but the two second-messenger systems appear to target the same K⁺ channels to mediate sweet transduction¹¹.

What, then, is the likely role of gustducin here? Again, as with bitter tastes, the $\beta\gamma$ subunits of gustducin may stimulate

PLC in response to synthetic sweeteners. If this were the case, only the transduction of synthetic sweeteners should be affected in the knockout mice, but in fact they were no better at detecting sucrose than synthetic sweeteners. Participation of gustducin in sucrose transduction would mean that gustducin must inhibit a PDE rather than activate it, in what would be a new signalling pathway for the transducins.

A concern with all knockout experiments is what secondary effects might result from the absence of the protein in question. In the case of gustducin, only the α -subunit is absent in the null mice of Margolske *et al.*, which may leave excess uncomplexed $\beta\gamma$ subunits in the cells. Could excess $\beta\gamma$ activate PLC in the absence of taste stimuli? This would probably diminish responses to both sweet and bitter tastes. It is also possible that, in the absence of α -gustducin, basal activity of taste-cell PDE is diminished. This could increase levels of cAMP, causing sweet-sensitive taste cells to be in a constant state of adaptation.

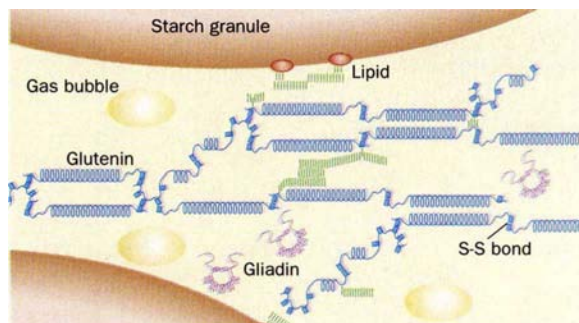
BIOPOLYMERS

Giant proteins with flour power

Colin W. Wrigley

THE largest protein molecules in nature occur in wheat flour, according to analyses of gluten protein by field-flow fractionation reported in two recent papers in the *Journal of Cereal Science*^{1,2}. The unique suitability of wheat flour for bread-making may depend on the enormous size of these wheat glutenin polymers, which have relative molecular masses ranging up into the tens of millions.

The longest single-chain polypeptide



The disulphide-bonded polymeric structure of wheat glutenin, which forms the protein matrix between the starch granules and gas bubbles in dough. (Original drawing by R. Appels.)

reported³ is a muscle protein called 'titin', which weighs in at about three million. Nucleotide sequencing of some fifty overlapping copies of titin's complementary DNA enabled its amino-acid sequence to be pieced together into a colossal chain of nearly 27,000 amino acids.

The unlikely similarity between titin (from muscle) and glutenin (from wheat flour) does not end with the consideration

If so, gustducin might be playing a modulatory rather than a direct role in transduction. Measuring resting levels of second messengers in taste cells from the knockout mice will diminish these concerns.

Moving on from their discovery that gustducin occurs in both sweet- and bitter-transducing taste cells, Margolske *et al.* have developed a line of transgenic mice in which the gustducin promoter also drives expression of β -galactosidase, which marks out gustducin-expressing taste cells by turning them blue or making them fluorescent (G. T. Wong and R. F. Margolske, unpublished results). This will allow gustducin-expressing taste cells to be targeted for physiological experiments, so that the role of gustducin can then be followed up. □

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of their enormous sizes. They also share the functional feature of 'springiness', generated by structural motifs in their repetitive domains. This spring is needed in muscle to enable it to return to its original position after extension, and it gives a mass of kneaded dough the bouncy viscoelasticity that will trap gas bubbles during the bread-baking process.

In the case of titin, the spring, which comprises 70 per cent of the motif in the centre of the polypeptide, is made up of a high proportion of the amino acids proline, glutamate, valine and lysine (PEVK in single-letter amino-acid code). This repeating PEVK motif⁴ appears in heart muscle as well as in skeletal muscle, although the PEVK region is only 163 amino acids long in the former.

The large central domain of the glutenin polypeptide is composed of repeating sequences rich in glutamine, proline and glycine. A spiral structure has been proposed⁵ for glutenin, consisting of inverse γ -turns, stabilized by extensive inter-turn hydrogen bonding through glutamine side chains.

Unlike titin, wheat glutenin proteins do not achieve their enormous sizes as single polypeptide chains. The glutenin proteins of gluten are disulphide-linked polymers of discrete polypeptides (see figure),

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which themselves range in size from about 50,000 to about 100,000. They constitute about half of the protein of wheat gluten. The other half consists of the gliadin fraction, a complex mixture of largely single-chain, hydrophobic polypeptides that have intramolecular disulphide bonds.

These two different types of dough proteins combine to give wheat dough its unique viscoelastic properties: the very large glutenin polymers contribute resistance to the extension forces of dough-mixing ('springiness'), while the gliadin monomers provide a plasticizer function. These properties are exploited in the production of bread, noodles, pasta and other wheat-based foods.

The very size of the glutenin polymers makes them insoluble in water, and presumably it is this characteristic that is critical for the wheat seed — insolubility means longer retention of precious protein supplies for germination, when these enormous storage proteins start to serve the purposes for which they were laid down by the ripening grain. The springiness of glutenin proteins may be an unexpected consequence of the structure that has evolved to maximize packing efficiency in the seed's storage endosperm.

Because of their unwieldy size, the characterization of these glutenin proteins has been particularly difficult. These most recent estimates^{1,2} have been obtained by a relatively new method known as field-flow fractionation (FFF), which has been most successful with entities that could be better classed as particulate rather than molecular. The FFF method⁶ depends on a cross-field to provide size-based fractionation of particles along a thin channel, giving retention times that translate directly into hydrodynamic diameters. The FFF results relate realistically to earlier relative molecular mass estimates⁷ of over twenty million from gel filtration of glutenin protein.

The 'cereal' story continues next September, when the international science community will have the opportunity to meet in Sydney, Australia, to discuss gluten chemistry and function at the sixth of a three-yearly series⁸ of International Gluten Workshops, called to focus attention on these enormous, but everyday, proteins. □

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The lights shineth in darkness

Joseph E. Borovsky

MY mother Janice grew up in Michigan, where she was often frightened by the northern lights, which chased her home on dark evenings. Do these mysterious emanations lurk behind the daylight sky? People such as young Janice will be relieved to know that they tend not to, as Newell and colleagues report on page 766

that, all other conditions being the same, auroral arcs are three times more likely to occur if the ionosphere below the satellite is in darkness than if it is sunlit. This finding seems to be contrary to expectations of auroral conjugacy³⁻⁵ — that is, that auroral arcs occur simultaneously on both the Northern and the Southern Hemisphere footprints of a magnetic-field line. However, because of viewing limitations, conjugacy of the aurora has been checked only when both footprints are in darkness.

Newell *et al.* contend that auroral conductivity is the critical factor in determining whether or not auroral arcs occur. They find a great asymmetry in the occurrence of arcs over dark versus sunlit ionospheres, and also point to the well-known asymmetry in the occurrence of auroral arcs in the region before local midnight as opposed to that after local midnight. Both of these asymmetries are consistent with asymmetries in the conductivity of the ionosphere: sunlight produces higher conductivity than is found in darkness and hot electrons from the midnight magnetosphere produce more conductivity in the ionosphere post-midnight than pre-midnight.

The two similar patterns (auroral occurrence and ionospheric conductivity) found in darkness and hot electrons from the midnight magnetosphere produce more conductivity in the ionosphere post-midnight than pre-midnight.

The two similar patterns (auroral occurrence and ionospheric conductivity)

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Pekka Parvainen/Science Photo Library

The aurora borealis, the visible manifestation of a high-energy electron current flowing from the magnetosphere to the ionosphere (where these displays occur, 100 km or so above the ground). It had been assumed that aurorae were equally common by day as by night, but Newell *et al.* have shown otherwise — night-time aurorae are much more common than daytime ones. Does that mean that the ionosphere controls the occurrence of aurorae, or is its interaction with the magnetosphere more complicated?

of this issue¹. For the scientist, this finding that auroral arcs are mostly absent in sunlight is surprising and may provide an important clue to their origin.

A long-standing problem in magnetospheric physics is the mechanism behind auroral arcs — the thin, dynamic curtains of light in the upper atmosphere caused by the impact of energized electrons travelling down the Earth's magnetic-field lines from the magnetosphere. A strong electrical current flows in and around an arc, and so it is reasoned that arcs are a manifestation of an electrodynamic coupling between the magnetosphere at high altitudes and the ionosphere at lower altitudes. A substantial fraction of the energy delivered to the Earth's magnetosphere by the solar wind is dissipated in auroral arcs and their associated ionospheric currents, yet there is no widely accepted physical picture of how arcs work and why they occur.

Newell and colleagues' findings come from a nine-year, five-satellite database² of electron-acceleration events (arcs) as detected by the DMSP (Defense Meteorological Satellite Program) satellites in polar orbits at heights of 835 km. The statistical analysis of these data shows

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led Newell *et al.* to conclude that a high conductivity of the ionosphere will preclude the formation of arcs, in support of a model wherein the ionosphere controls the auroral-arc current⁶⁻⁸. They further conclude that "discrete aurorae can now be understood to be the means whereby the ionosphere establishes the conductivity needed to support currents between the magnetosphere and ionosphere". This may be so.

Still, many researchers believe that auroral arcs originate because of limitations on the ability of the magnetosphere to carry current⁹⁻¹¹, not because of the current-carrying ability of the ionosphere. According to various arguments¹²⁻¹⁴, electric fields arise in the magnetosphere when the current density therein becomes too high; magnetospheric electrons that are accelerated by these electric fields then produce visible auroral arcs when they hit the atmosphere. It is known that

the interplay between the current-carrying abilities of the magnetosphere and ionosphere can lead to preferred thicknesses of auroral structures¹⁵⁻¹⁷ (although the thicknesses associated with this interplay tend to be much greater than those of auroral arcs); perhaps the thickness of the magnetospheric current layer is affected by the ionospheric conductivity, and so it might follow that the current density in the magnetosphere also is affected by that conductivity.

Time will tell whether auroral arcs are created to produce ionospheric conductance rather than to produce magnetospheric conductance. Newell and colleagues' findings should help to force the issue. □

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MALE STERILITY

Fruit(less)flies provide a clue

Paul S. Burgoyne

ALTHOUGH species-hopping is now commonplace for molecular geneticists, large leaps still engender excitement because they imply the identification of gene functions that are of fundamental importance. On page 783 of this issue, Eberhart, Maines and Wasserman¹ report the characterization of a *Drosophila* male-fertility gene, *boule*, which is clearly related to the human gene *DAZ* — for *Deleted in Azoospermia* — a candidate male fertility gene located on the Y chromosome.

It was twenty years ago that Tiepolo and Zuffardi reported finding six men with no sperm in the ejaculate (azoospermia) and who had deletions of much of the Y-chromosome long arm. This led them to propose that "factors controlling spermatogenesis" were located in a region (Yq11) of the long arm². The term azoospermia factor (*AZF*) was subsequently coined as a genetic 'label'.

The first molecular foothold was provided by the identification of human chromosome-Y DNA sequences with similarities to *Drosophila* chromosome-Y fertility genes, which preferentially mapped into the *AZF* region of the human Y (ref. 3). However, the significance of this observation is unclear. After further refinement in the mapping of the *AZF* region with the help of sterile males with microdeletions in Yq11, two papers appeared that each heralded 'candidate' genes for *AZF*. The first described the identification of a gene family that encoded proteins with RNA-binding motifs (originally given the label *YRRM*, but now relabelled *RBM*, for *RNA-binding-motif*

gene family)⁴. The second paper reported the identification of *DAZ* (for which a *Drosophila* homologue is now reported), which also encodes a presumed RNA-binding protein⁵.

With two candidates up for election, how do you decide? Both genes are expressed in testes, as befits their proposed function, but proof is no easy matter. One increasingly popular approach is to identify and mutate by 'gene targeting' the homologous gene in the mouse. This approach has been attempted for both candidates. In the case of *RBM*, it was thought that there was a single Y-linked copy in mouse which should be amenable to gene targeting, but when the mouse gene was cloned, it was also found to be present in multiple copies⁶. In the case of *DAZ*, no mouse Y-linked copy could be identified.

Given the intensity with which proponents support their candidates, the work of Eberhart *et al.* identifying a *DAZ* homologue in *Drosophila*, with attendant proof of spermatogenic function, will probably be greeted with delight in the *DAZ* camp and with dismay by *RBM* followers. However, what appears to be the obvious conclusion — that *DAZ* is a spermatogenesis gene with a long evolutionary pedigree which can be equated with *AZF* — is less obvious than it first appears.

First of all, the protein with the closest structural similarity to the *Drosophila* *boule* gene product is, in fact, encoded by the mouse gene *Dazla*⁷ (located on mouse chromosome 17), which was identified during the search for a mouse Y homo-

logue of *DAZ*. On the basis of comparisons between the mouse and human gene maps, we can be virtually certain that there is a *Dazla* homologue on the short arm of human chromosome 6 (known as an autosome as it is not a sex chromosome). The existence of a human autosomal homologue is supported by the hybridization of a *Dazla* probe to human female DNA (as females lack a Y chromosome). Indeed, the presence of a Y-chromosomal *DAZ* gene appears to be limited to primates⁷.

Boule, like *Dazla*, is located on an autosome, so the evidence in fact suggests that there is an autosomal spermatogenesis gene with a long evolutionary history, and that much more recently (during primate evolution) a copy has moved onto the Y chromosome. This copy then diverged, mainly by amplifying a region encoding 24 amino acids, generating the so-called '*DAZ* repeat'. Further *DAZ*-related transcripts have now been identified with different numbers of '*DAZ* repeats', and it is possible that these are encoded by a separate *DAZ*-related gene (or genes) with the competing gene designation *SPGY* — for spermatogenesis gene on the Y chromosome⁸.

What, then, are the implications of the paper by Eberhart *et al.*? First, it is clear that *boule* is a gene that is essential for spermatogenesis (but not oogenesis) in *Drosophila*. In this regard, it is similar to the *Drosophila* gene *Rb97D*, which also encodes a putative RNA-binding protein and is essential for male, but not female, fertility⁹. The equivalent mammalian gene is represented by the mouse gene *Dazla*, and we can look forward to its targeted mutation to determine whether it is also essential for spermatogenesis. We can also be sure that the human autosomal copy has or will soon be cloned, and it will be intriguing to see if it is still functional.

What, then, of the *AZF* candidates? Evolutionary biologists have long argued that the Y chromosome will 'attract' fertility factors, and I have always held the view that a gene on the Y chromosome that is expressed exclusively in spermatogenic cells will unquestionably play a role in spermatogenesis. On this basis, both *RBM* and *DAZ*/*SPGY* should function in spermatogenesis. However, that is not to say that they will necessarily have essential functions. Spermatogenesis is at the sharp end with respect to selection, and any Y gene that even slightly improves the efficiency of

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the process will be strongly selected for.

Increasing the production and specificity of RNA-binding proteins, which are involved in RNA transport and processing, would certainly be one route to improved efficiency. In this regard, it is worth noting that *DAZ* has very shaky credentials as an essential spermatogenesis gene. Reijo *et al.*⁵ reported a variable phenotype in men lacking *DAZ*, with two even having "occasional mature, condensed spermatids" (sperm to you and me), and now Vogt *et al.* report the transmission of a *DAZ/SPGY* deletion from father to son¹⁰. Deletion analysis of the mouse *RBM* homologues also points to *RBM* being involved in,

but not essential for, the spermatogenic process (P. S. B. *et al.*, unpublished).

So perhaps we should all heed the view of Peter Vogt and his colleagues, who have argued that there are at least three distinct spermatogenic factors that map into the *AZF* interval, only one of which gives an unequivocal spermatogenic failure (manifest as a 'Sertoli cell only' phenotype)¹⁰. If this is true, the *AZF* region has still to yield up the main culprit. □

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VERTEBRATE PALAEONTOLOGY

Early amphibian globetrotters?

Andrew Milner

THE first third of the history of tetrapods (four-legged vertebrates) is the most poorly known third, and the fossil record is still sufficiently patchy for us to be deceived by the patterns we see. The interval from the first appearance of tetrapods in the Late Devonian (377 million years ago) to the global tetrapod faunas of the Middle Permian (256 million years ago) occupies a span of about 120 million years. Since the consensus on continental drift in the mid-1960s, almost all our information on this major phase in terrestrial vertebrate history has been derived from one palaeo-continent: Euramerica (now North America, Greenland, and Europe west of the Urals). In this region, the Upper Devonian is represented by stem-tetrapods from Greenland, Russia, Latvia and North America, and the Carboniferous and Lower Permian by coal-swamp, lake and flood-plain amphibian and reptile faunas from North America and Europe.

This sequence of early tetrapod faunas has become the surrogate for the

sequence of faunas throughout the world. Until the early 1970s, there was no reliable evidence of any tetrapods outside Euramerica during this time, despite rich faunas of earlier and later ages, and it was assumed, not unreasonably, that the origin and early evolution of tetrapods was restricted to Euramerica¹. The new Carboniferous tetrapod material from Australia reported on page 777 of this issue by Thulborn and colleagues² is the latest in a series of antipodean discoveries that have completely changed this perspective.

Since 1972, discoveries in Australia (eastern Gondwana in the Late Palaeozoic) of Upper Devonian trackways³, an Upper Devonian tetrapod-like jaw⁴ and some possible Middle Devonian trackways⁵ have generated a shift in our view of the geography of tetrapod origins. Initially, there was a direct switch to suggesting an entirely East Gondwanan origin for tetrapods⁶⁻⁸ instead of a Euramerican origin, but the recent reassessment of the Euramerican panderichthyid fishes as tetrapod relatives⁹, and the recognition of

the Elginerpetontidae (also Euramerican) as a family of stem-tetrapods¹⁰, has neutralized that argument.

It now seems likely that the development of tetrapod morphology and the water-land transition probably took place across an area not less than the entire continental equatorial belt. However, the apparent absence of Carboniferous tetrapods in Australia, combined with East Gondwana's southward drift during the Carboniferous into the zone of the Late Palaeozoic glaciation, had continued to suggest that the early tetrapods of East Gondwana were frozen out of existence before they could diversify, and that, even though tetrapod origins were pan-equatorial, the subsequent diversification was still an exclusively Euramerican phenomenon.

The new Australian material reported by Thulborn *et al.* shows that at least three types of tetrapod were present there in the Lower Carboniferous, including the family Colosteidae, and that both stem-amphibians and stem-amniotes were probably present. This new material thus demonstrates that not only did the appearance of structural tetrapods and the water-land transition take place right across the equatorial belt, but so, too, did the early phases of the subsequent diversification.

The significance of this change in perspective is twofold. First, it forces the conclusion that Early Carboniferous tetrapods ranged throughout equatorial northern Gondwana. The recognition of one family — the Colosteidae — shared between East Gondwana and Euramerica demonstrates this. Thus, Early Carboniferous tetrapods might now be expected as fossils in several regions (Turkey, Arabia, Iran, Tibet) where their presence has not yet been demonstrated (Fig. 1a). Second, by dramatic extension of the range over which tetrapod diversification occurred, it opens up the possibility that this phenomenon had a geographical component.

Until now, we have tended to assume that the diversification of tetrapods,

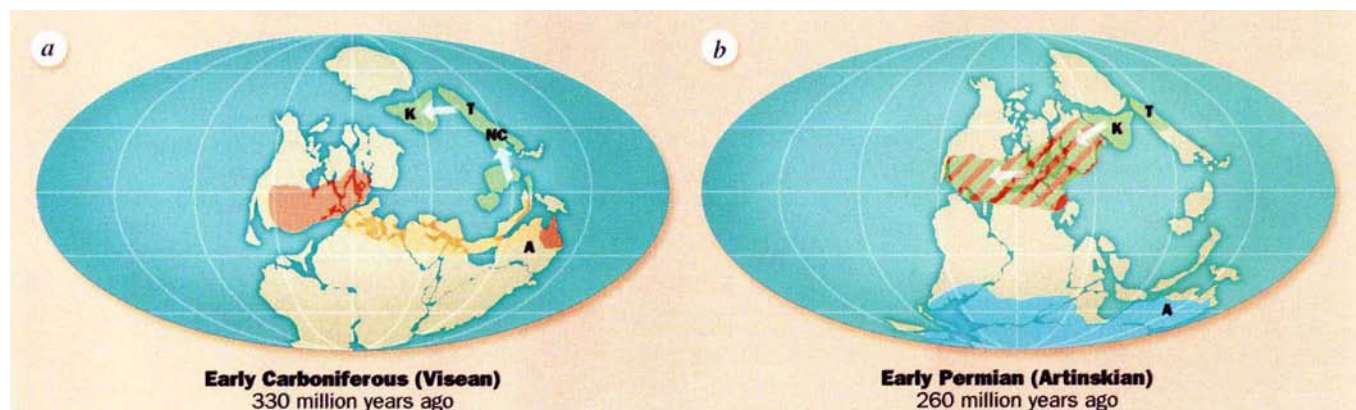


FIG. 1 a, Early Carboniferous world map showing known distribution of tetrapods (orange), inferred equatorial range (yellow), and possible range extension of seymouriamorphs onto Asian plates (green and arrows). A, Australia; K, Kazakhstan; NC, North China; T, Tarim. b, Early Permian world map showing minimum known extent of Edaphosaur-Nectridean faunal province (orange), range of seymouriamorphs (green) and inferred path of their range extension (arrows). The extent of the Late Palaeozoic ice cap is indicated in blue. (Maps after ref. 16.)



FIG. 2 *Discosaurus*, a small, probably larval seymouriamorph from the Lower Permian of the Czech Republic. Calibrated scale bar, 5 cm. (Science Museum of Minnesota; photograph by A. M.)

apparently centred on equatorial Euramerica, was largely a process of ecological niche-hopping within a single continent. The new material raises the possibility that different tetrapod groups diversified in parallel in different regions of the equatorial belt and subsequently extended their geographical ranges, interacting with each other as they did so. Given that we have a reasonably coherent Carboniferous-to-Lower Permian record only in Euramerica, we might look to it to provide circumstantial evidence of immigration of groups from elsewhere. This would manifest itself as the sudden appearance in the fossil record of abundant or diverse distinctive groups with no apparent antecedents or close relatives within Euramerica. The tetrapod group that most plausibly fulfils this criterion is the Seymouriamorpha.

The seymouriamorphs are agreed to be close relatives of the true amniotes, although they retain gill-bearing larvae and lateral-line canals. The best-known genus, *Seymouria*, was for many years considered to be the earliest reptile. Well-dated seymouriamorphs first appear suddenly and simultaneously in the earliest Permian of Euramerica (New Mexico, Utah, Texas, France, Germany and the Czech Republic), with no Late Carboniferous relatives whatsoever. Their consistent position on recent cladograms (see ref. 11, for example) implies that they were already been present in the Early Carboniferous, and yet several rich Late Carboniferous vertebrate assemblages in Euramerica include relatives of almost every other Early Permian group, but no seymouriamorphs. Not only this, but populations of seymouriamorph larvae ('discosauriscids'; see Fig. 2) are found in Permo-Carboniferous beds of imprecise age on the Kazakhstan and Tarim continental plates, in strata where no other tetrapods have been found. Several of these central Asian 'discosauriscid' genera (*Ariekanerpeton*¹², *Urumqia*¹³, *Utegenia*¹⁴) have been named, and one of them, *Utegenia*, appears to be one of the most primitive seymouriamorphs¹⁵.

An orthodox interpretation might be that seymouriamorphs filled a niche that made their preservation unlikely until the beginning of the Permian, at which point taphonomic circumstances changed and they were suddenly abundantly preserved, both in Euramerica and in those plates newly accreting to Euramerica in the Lower Permian. However, with tetrapods also diversifying in East Gondwana in the Early Carboniferous, it is equally possible that seymouriamorphs first

appeared there and, instead of spreading westwards, spread or hopped north across the chain of North China, Tarim and Kazakhstan plates during the Upper Carboniferous (Fig. 1a), bursting onto the Euramerican scene in the Early Permian when the Kazakhstan plate finally sutured with Euramerica⁸ (Fig. 1b). These alternatives are potentially testable, depending on where well-dated Carboniferous seymouriamorphs are ultimately demonstrated to have occurred. □

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Erratum

In Laurence Hurst and Gilean McVean's News and Views article published last week (*Nature* **381**, 650–651; 1996), dealing with asexuality in endosymbiotic bacteria, the bars in the diagram were incorrectly described in the caption. Blue shows the ratio of mean non-synonymous to mean synonymous substitutions per nucleotide site in the endosymbiont *Buchnera*; red the ratio in the free-living enterics *Escherichia coli* and *Salmonella typhimurium*.

DAEDALUS

Noise annoys not

THESE days, our ears are too sensitive for their own good. Primitive tribes, with only natural sounds to listen to, retain their acute hearing into late age. Modern civilization, however, batters our ears into early deafness. They have a natural protection mechanism, but it badly needs upgrading.

It uses two tiny muscles, the tensor tympani and the stapedius, which reduce the ear's sensitivity by stiffening the joints of its transmission bones. They are tensed automatically by loud noise. They worked well in prehistoric times, when most noises built up slowly. But they cannot react fast enough to modern bangs and crashes, and sustained uproar fatigues them. Daedalus wants to warn them of noise in advance.

A sound wave launched upwards into the atmosphere, he notes, can be tracked by radar. The density gradient of the wave reflects the radar beam. So he is scaling the idea down. He is devising a little diode-radar placed next to the ear, which scans the nearby air for the density signature of an approaching noise. A mere metre of range would give 3 milliseconds of advance warning, ample time to tighten the defending muscles against the coming crash.

At first Daedalus intended to tense the muscles with a small electric shock, but soon realized that this would be more distressing than the noise itself. He then recalled the odd fact that many people can hear audio-modulated microwaves. The tissues of the ear must be electronic rectifiers, able to demodulate a high-frequency carrier. Now high-frequency currents are not painful. So Daedalus's 'Radar Earplugs' will react to loud sound by injecting a painless high-frequency current into each ear, via electrode pads strategically placed around the ear. The ear's gain-control muscles nestle in insulating corridors of bone. Proper siting of the pads could funnel their current largely through those muscles. They will rectify the current and tighten in the resulting d.c., thus turning down the gain before the racket arrives. They will keep it down as long as necessary — long after the nerve serving them would have succumbed to fatigue.

Radar Earplugs will be small and neat enough to masquerade as earrings, or fit on spectacle frames. You will hardly notice their action; your hearing will seem just as acute. Yet sudden loud sounds will cease to startle you, and sustained cacophony will seem far less fatiguing. Wear them regularly, and you will retain pin-sharp hearing well into senility — unless you foolishly bypass your own defences with a personal stereo.

David Jones

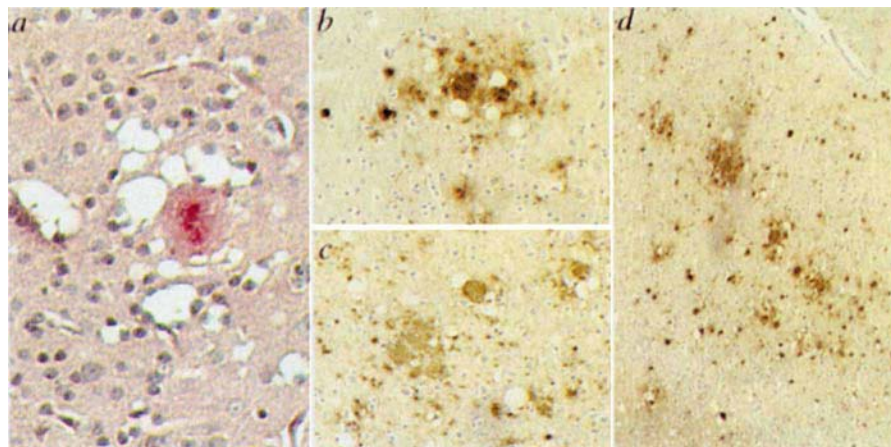
BSE transmission to macaques

SIR—The recent description in the United Kingdom of a new variant of Creutzfeldt–Jakob disease (vCJD) with a unique clinicopathological presentation has raised concern about a possible link with the epizootic disease bovine spongiform encephalopathy (BSE) in British cattle¹. Twelve cases of vCJD were characterized by their young age and by an unusual and remarkably uniform pathology^{1,2}; spongiosis was most evident in the striatum and thalamus, and was present focally in cortical areas and in the cerebellum. The most consistent feature was the presence of kuru-type plaques surrounded by vacuoles referred to as “florid” plaques³. These observations suggest that vCJD has a common origin. Here, we report that the pathological ‘signature’ of the BSE agent in cynomolgus macaques is identical to that of vCJD in humans.

In this study, two adult and a neonate cynomolgus macaques were inoculated by an intracerebral route with 400 and 200 μ l respectively of a 25% BSE cattle brain homogenate from the United Kingdom: 150 weeks after inoculation, the two adults developed abnormal behavioural signs including depression for the one, edginess and voracious appetite for the other. Rapidly, both developed cerebellar signs with truncal ataxia, broad-based gait and tremors, as well as intermittent priapism. Ataxia became extremely severe within a few weeks and the animals had to grasp the cage bars to maintain an upright posture. As the disease progressed, the behavioural changes worsened and myoclonic jerks were seen. The adult macaques were killed 10 and 11 weeks after the onset of behavioural symptoms. The youngest animal exhibited similar clinical signs 128 weeks after inoculation and was killed 23 weeks thereafter at the same clinical stage as the adults.

Immunoblot analysis revealed that the amounts of abnormal prion protein (PrP^{RES}) accumulation in different brain regions were strikingly similar in the three animals and correlated with the deposition pattern described in vCJD¹, with more PrP^{RES} in the cerebellum than in the forebrain (data not shown). Spongiform

change and astrogliosis were most intense in the thalamus and the striatum, but were also severe in the granular and molecular layers of the cerebellum and in the cerebral cortex, where the vacuoles exhibited a patchy distribution. The parahippocampal gyrus and the cingulate gyrus were always severely affected. The hippocampus was almost spared, and the brain stem was moderately affected in the tegmentum.



Cerebral cortex of the youngest monkey. *a*, PAS staining of a florid plaque; *b–d*, PrP immunohistochemistry showing pericellular deposits, kuru-type PrP plaques associated with vacuoles (*b*) and larger uni- or multicentric plaques (*c*). These plaques were also seen in the cerebellum, together with multiple smaller plaques in the granular layer. Immunostaining in the thalamus revealed intense widespread granular and perivacuolar deposition, as well as linear tract-like deposits within the grey matter. PrP immunostaining was performed with the monoclonal antibody 3F4 according to a method previously described⁸.

Numerous plaques were easily demonstrated using PAS and Alcian blue stains (*a* in the figure). They consisted of a central eosinophilic dense core surrounded by a pale periphery and, beyond this, a ring of vacuoles was seen in many instances. Such lesions, sometimes grouped into small clusters, were indistinguishable from the florid plaques described in vCJD^{1,2}. They were most severe in the young macaque. PrP immunohistochemistry confirmed the extensive distribution of these plaques and revealed the presence of the different types of PrP deposits described in vCJD (see figure). Moreover, the regional distribution of the different types of PrP deposits was similar in all monkeys and similar to that observed in the human cases of vCJD.

This neuropathological phenotype was different from that observed in two macaques inoculated with sporadic CJD (L. Court, F. Cathala), where no plaques were seen. In the latter, spongiform changes consisted of smaller and more diffuse vacuoles. Lesions were most numerous in the cortex, and no vacuoles were seen in the cerebellum (data not shown).

The level of congruency between the host and the donor PrP is crucial to the species barrier effect⁴. Cynomolgus macaques belong to Old World monkeys, which represent the evolutionary nearest available experimental model to humans, and whose PrP has a high degree of homology with that of man (96.4% for the cynomolgus macaque⁵). Furthermore, the codon 129 of the PrP gene, which is polymorphic in humans, is important in the susceptibility to CJD⁶. It may therefore be important that this

codon is monomorphic for methionine in cattle and in cynomolgus macaques⁵, and that all vCJD cases reported were homozygous for methionine^{1,2}.

The macaques in our study were inoculated intracerebrally, whereas the hypothesis of a human contamination with BSE implies mainly an oral route of infection. However, as the BSE agent consists of a single and stable strain, it is most likely that the pathology at the terminal stage of disease does not depend upon the inoculation route, as it has been demonstrated in experimental stabilized strains including mouse BSE⁷.

Taken together, the similarity of the clinical, molecular and neuropathological features observed in the three BSE-infected macaques with the human cases of vCJD is striking, particularly the distribution of spongiform changes and the

This Scientific Correspondence article is on *Nature's* web site at www.nature.com. See also the News and Views article by Adriano Aguzzi on page 734 of this issue.

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typical morphology of the plaques. This study provides evidence supporting the hypothesis that the BSE agent is responsible for the emergence of the new form of CJD in humans.

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WW domains and retrovirus budding

SIR — It has long been known that retroviral Gag proteins, in groups of roughly 2,000, can direct the budding of enveloped particles at the plasma membrane. Although many of their functional domains have been identified, an understanding of how Gag proteins interface with the host cell remains a mystery. Interactions with cellular proteins are thought to be essential as budding does not require any of the other virus-encoded products (for example Pol or Env). We wish to report an observation that strongly suggests a role for host proteins in the final stages of virus release.

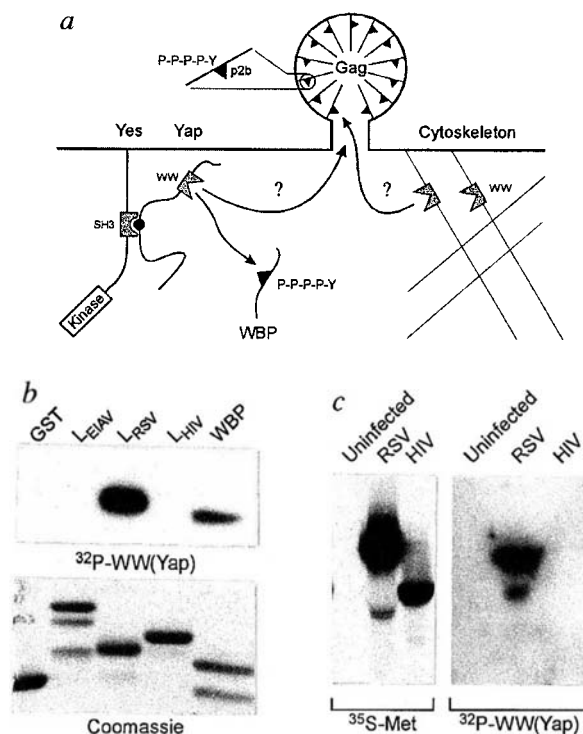
One of the functional domains of Gag, the L domain, operates late in budding. Deletion of L results in an accumulation of molecules in virus-like structures that fail to separate from the cell surface. The first L domain to be identified was that of HIV (human immunodeficiency virus)¹. It is located near the carboxy terminus of Gag, in the p6 sequence, and its critical amino acids include P-T-A-P. In contrast, the L domain of Rous sarcoma virus (RSV)

Gag is near the amino terminus² in the p2b sequence and its critical residues are P-P-P-P-Y³. This sequence is not found anywhere in HIV Gag; likewise, the sequence of the HIV L domain is not found in RSV Gag. The only other identified L domain is that of equine infectious anaemia virus (EIAV), near the carboxy terminus of Gag (within the p9 sequence) but otherwise resembling neither P-T-A-P nor P-P-P-P-Y⁴.

L mutants of RSV can be restored to budding competence by attaching the L domain of HIV or EIAV to their carboxy termini; thus, L functions are exchangeable between retroviruses of different genera⁵. Surprisingly, the L domains of RSV and HIV can also function when moved to ends of Gag opposite their normal location; hence, these functions are positionally independent, too^{3,4}. The means by which L domains mediate the pinching-off step is not known, but a recent clue concerning the yes oncogene is intriguing.

The Yes protein tyrosine kinase is located on the cytoplasmic face of the plasma membrane, the same surface to which Gag proteins are targeted (a in the figure). It contains an SH3 domain, which permits interaction with Yap (Yes-associated protein) during signal transduction⁶. Yap contains a 'WW' motif — a sequence of 38 amino acids containing two widely spaced tryptophans. This recently discovered motif is found not only in signalling molecules like Yap but also in certain regulatory and cytoskeletal proteins⁶.

Two Yap binding proteins (WBP-1 and WBP-2) have been found by using glutathione S-transferase-WW (GST-WW) fusion proteins to screen a complementary DNA expression library⁷. The sequences of these proteins are different except for the five residues that interact with the WW motif. Remarkably, the common sequence is absolutely identical to that of the RSV late domain: P-P-P-P-Y. This observation, in combination with the membrane location at which L mutants accumulate and the presence of WW domains in cytoskeletal proteins (which have been hypothesized to be involved in Gag-mediated budding), is a compelling argument for the involvement of a cellular protein, one with a WW



a, Gag proteins direct budding from the plasma membrane during retrovirus assembly. Interactions between Yes, Yap and WBP proteins during signal transduction take place on the same membrane, as illustrated. The L domain of RSV Gag (in p2b) has a sequence identical to the portion of WBP that interacts with a WW motif. b, We subjected native GST, GST fusion proteins containing each of the known L domains (L_{EIAV} , L_{RSV} or L_{HIV}) and GST-WBP-1 to SDS-PAGE. We either stained proteins with Coomassie Brilliant Blue R or transferred them to nitrocellulose and probed with ^{32}P -labelled GST-WW(Yap)⁷. c, We pelleted retroviral particles containing uncleaved RSV or uncleaved HIV Gag proteins from the medium of transfected COS-1 cells and performed SDS-PAGE. The release of particles was confirmed by labelling with ^{35}S -methionine²⁻⁴. We transferred unlabelled particles produced in parallel cultures to nitrocellulose, and probed them with ^{32}P -labelled GST-WW(Yap). We detected only the RSV Gag protein.

motif, in the late stages of budding for RSV. Because the L sequences of RSV, HIV and EIAV are distinct, there are likely to be at least three host proteins that can provide this function, although all may not contain WW.

To test the possibility that a WW domain might be involved in the RSV Gag late function, we examined the ability of GST fusion proteins containing the three known L domains to bind to the WW domain of Yap in a filter-binding assay. The WW domain bound very well to the L sequence of RSV but not to those of HIV or EIAV (b in the figure). Moreover, the WW domain also was able to discriminate between the full-length RSV and HIV Gag proteins (c in the figure). GST alone (negative control) and GST-WBP-1 (positive control) behaved as expected.

Is Yap the protein that interacts with the L domain of RSV *in vivo*? This is possible, but WW motifs are found in many proteins. Sequences flanking the motifs are likely to influence the specificity of binding (as they do for SH3 ligands⁸);

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hence, no assumptions can be made at this stage. In summary, each retrovirus may have evolved to take advantage of unique host proteins at the particular sites on the membrane where they assemble — just as they have evolved to use different receptors on the cell surface for entering host cells.

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Leptin activation in hypothalamus

SIR — The excessive fat deposition and other genetic disorders seen in the obese *ob/ob* mouse has been shown to be due to a mutation (premature stop codon) in the *ob* gene¹. Normally, the *ob* gene is expressed in adipose tissue, with the secreted circulating gene product, leptin, being thought to act as a hormonal feedback signal to regulate fat cell size via hypothalamic mechanisms controlling food intake and metabolic rate. Thus, treatment of *ob/ob* mice with leptin restores this signal and corrects most, if not all, of the mutant's metabolic and endocrine defects^{2–5}.

The leptin receptor (Ob-R) gene has at least six splice variants, but the observation that the Ob-Rb variant is highly expressed in hypothalamus, and that the obese diabetic *db/db* mouse mutation is

found in the Ob-Rb variant⁶, strongly suggests that leptin normally exerts its effects on this hypothalamic receptor. How this large (M_r 16,000) leptin protein crosses the blood–brain barrier to activate the Ob-Rb receptor is not known, and it is possible that the hypothalamic leptin receptor is in an area where there is a weak or non-existent blood–brain barrier. To obtain a more precise idea of the hypothalamic areas involved in mediating the effects of leptin, we used Fos protein immunoreactivity to localize expression of the immediate early gene *c-fos* as a marker of neuronal activation⁷.

The only hypothalamic area to show obvious, dense Fos protein immunoreactivity is the paraventricular nucleus in *ob/ob* mice treated 3 hours previously with leptin (see figure). There is no Fos protein staining in the paraventricular nucleus of lean mice receiving leptin, nor in *ob/ob* mice receiving vehicle (control group). In the *ob/ob* mice receiving leptin, there is little Fos protein immunoreactivity elsewhere in the hypothalamus (data not shown), other than some staining in the zona incerta and arcuate nucleus and a few scantily stained neurons in the ventromedial hypothalamus; we saw no staining in any hypothalamic area 24 hours after leptin injection.

The hypothalamic areas mentioned above are important in the neuroendocrine control of energy homeostasis, with the paraventricular nucleus being particularly prominent as a focus for the action of neuropeptide Y, corticotrophin-releasing factor and the monoamine neurotransmitters affecting ingestive behaviour and autonomic control of metabolism. The ventromedial hypothalamus is another important area controlling energy intake and expenditure, and we were surprised to find little or no evidence of *c-fos* expression (neuronal activation). However, it is possible that greater activation of this area (and others) might be seen at a later stage (after 3 hours but before 24 hours) after leptin injection, or with repeated leptin injections.

We believe that the failure of leptin to induce Fos protein immunoreactivity in the paraventricular nucleus of lean mice is because lean mice, unlike *ob/ob* mice, produce mature leptin and are therefore less sensitive to doses of exogenous leptin. However, a single injection at a tenfold higher dose (10 mg per kg) still fails to activate the paraventricular nucleus in lean mice, suggesting that even higher, and/or repeated, doses may be required.

In conclusion, it seems that the most rapid and strongest hypothalamic response to leptin in *ob/ob* mice occurs in the par-

aventricular nucleus, which suggests that this is where the Ob-Rb receptor is most likely to be located, although one cannot exclude a more distant location with projections to this hypothalamic area.

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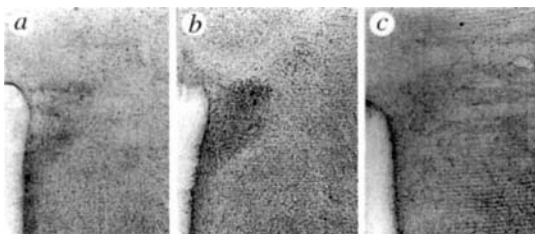
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Flashing males win mate success

SIR — The spectacular bioluminescent displays of male fireflies (Coleoptera: Lampyridae) are widely regarded as advertisement signals under sexual selection pressures¹. However, despite extensive work on species identity², mimicry³ and synchrony⁴ in bioluminescent signalling systems, potential influences of intra- and intersexual selection on the evolution of flash characters have never been demonstrated in any species. Here, we report the first evidence for intersexual selection on a bioluminescent signal character. Using a novel photic playback experiment, we determined that females of the Nearctic firefly species *Photinus consimilis* prefer flash rates that exceed the mean rate in the male population but prefer flash lengths that approximate the mean. This combination of directional and stabilizing selection indicates that females do not simply choose signals containing high photic power.

The *P. consimilis* signalling system includes elements typical of many Lampyridae⁵. Males signal while slowly flying 1–3 m above the ground. A stationary female on the ground which detects a male's signal may reply with a dimmer signal after a characteristic delay; this often attracts the male to her vicinity. A signalling dialogue may ensue and culminate in courtship and mating.

Male signals are 0.7–3.1-s flash 'trains' given at 2.5–4.0 trains per min. The males fly 3–6 m between producing successive flash trains, but usually hover while flashing. Each flash in a train is approximately 70 ms long (*F*). Radiant intensity during a flash rises gradually to a plateau, then slowly decays. Flash period (*T*) and inter-flash interval (*T-F*) within a train are influenced by temperature and may be predicted by least-squares linear models; the average male flash rate ($\bar{T}^{-1}$) at



Fos protein immunoreactivity in the paraventricular nucleus of a lean mouse treated with leptin (a), an *ob/ob* mouse treated with leptin (b) and an *ob/ob* mouse treated with vehicle (c). Female *ob/ob* obese and lean (+/?) mice were injected with 1 mg kg⁻¹ recombinant murine leptin (Amgen) or vehicle, and 3 h later were overdosed with sodium pentobarbitone and immediately transfused transcardially with ice-cold saline, followed by 4% paraformaldehyde. The brains were sliced in 100-μm-thick coronal sections through the hypothalamus and incubated in primary Fos antiserum (OA11-824; Cambridge Research Biochemicals). We used an avidin–biotin–horseradish peroxidase procedure, with diaminobenzidine as a chromogen⁸, to visualize Fos protein immunoreactivity.

23.3 °C is 3.3 s⁻¹ ($n=61$). Female signals consist of 1–12 flashes which begin 6–10 s after the end of a male's flash train. At 23.3 °C, the female flash rate is approximately 2 s⁻¹ ($n=28$).

Contrary to the conventional wisdom that flash characters are fixed entities within firefly species⁶, we found that temporal characters in *P. consimilis* signals varied considerably between males. We video-recorded flash trains from 61 males flying at Roaring River State Park, Missouri, corrected their flash rates, and consequently their inter-flash intervals, to values predicted for 23.3 °C, and calculated mean values for each character of each individual (Fig. 1).

Because of the marked variation in all temporal characters, we investigated whether female *P. consimilis* evaluate the flash characters of potential mates and thereby discriminate among them. Using a photic analogue of the acoustic playback experiment, we tested the responses of females to simulated male signals in which intensity, and spectral and temporal characters were controlled (Fig. 1). We held peak radiant intensity and wavelength constant for every trial, and adjusted values of temporal characters to one of five levels spanning the range displayed by the male population at the temperature of the trial.

To test the influence of number of flashes per train (N) on female response, we fixed all other temporal characters at levels average for the population and recorded replies to the five selected N levels: 2, 4, 6, 8 and 10 flashes. We detected no differences between the proportions of females replying to the various N levels. However, the mean frequency with which females replied to the lowest N level and the number of flashes included in those replies are significantly lower than in

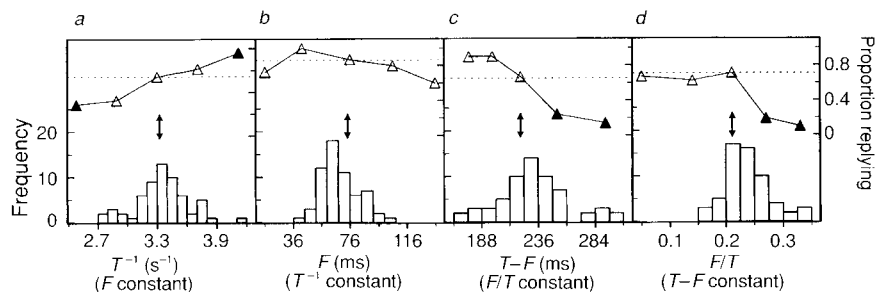


FIG. 1 Between-male variation and female preference for 4 male signal characters in *Photinus consimilis*: a, Flash rate, T^{-1} (T =flash period) ($n=22$); b, flash length, F ($n=15$); c, inter-flash interval, $T-F$ ($n=21$); d, duty cycle, F/T ($n=21$). Histograms, proportions of males whose mean temperature-corrected values for a signal character fell within the given bins. Arrows, population means. Triangles, proportions of females ($n=15-22$) that exhibited one or more flash replies to simulated male signals with the given level of the character; proportions that differed significantly ($P<0.05$; G-test, with Holm correction for multiple tests) from proportion responding to the mean level (dotted line) are shown solid. Simulated signals were created and edited by computer, transferred to digital audio tape (DAT), and played back through a frequency-to-voltage converter at 3.5 trains per min; their voltage was used to drive photic output of a light-emitting diode (LED = 580 nm) positioned 1.5 m above the female.

replies to the mean N level.

Testing the influences of the other temporal characters was more difficult. Only one character could be fixed at a time, because flash rate, inter-flash interval, flash length and duty cycle (F/T) are interdependent. If, for example, flash length were fixed, a heightened response to shorter inter-flash intervals might reflect preference for higher flash rate or higher duty cycle. Thus, in order to determine the influence of any single character on female response, it was essential to combine results from tests of all four characters. We therefore conducted four series of trials, in each series fixing one character (and $N=6$) at the level average for the population. We presented 1 character in each series at five selected levels; the two remaining characters varied necessarily. Our tests revealed significant differences between the proportions of females replying to the levels of each variable character except flash length (Fig. 1). Flash rate was the fixed character in the series of trials wherein flash length was the designated variable (Fig. 1b); absence of differential responses only in this series indicated that females were influenced primarily by flash rate. But female replies to very brief and long flashes are both less frequent and include fewer flashes than do replies to the mean male flash length (Fig. 2).

Results from the photic playback experiment demonstrate strong directional selection favouring rapid male flash rates in *P. consimilis*. Females are more likely to reply to simulated male flashes delivered at faster rates, and their replies to the faster rates include more flashes (Fig. 2). Because visible female replies are an essential prelude to pair formation, a male's mating success and fitness should

be proportional to his flash rate, provided that his flash number exceeds a minimum value and his flash length approximates the mean.

Neither flash number nor flash length is correlated with flash rate ($P>0.10$) in signals recorded from the male population. Consequently, female preference for high flash rate is unlikely to select indirectly for other signal features. Although the striking discrepancy between the average and preferred male flash rates (Fig. 2) indicates that such female preference is strong, our data alone cannot support any one sexual selection model over another.

The finding that female *P. consimilis* identify male flashes based on their rate but not their power indicates the operation of a perceptual filter yielded by phasic neural responses to flash onsets, followed shortly thereafter by adaptation and inhibition⁷. Such neural processing may be an ancestral feature. Moreover, this feature could represent an evolutionarily conservative mechanism in neural systems⁸, which could account, in part, for some astonishing similarities in female choice for rapid rhythms observed among diverse species in pulsing visual, acoustic⁹ and now photic signals.

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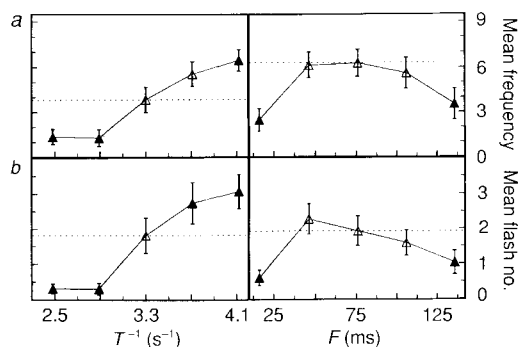


FIG. 2 Frequency and number of flashes in female *P. consimilis* replies shown in Fig. 1 (a, b). a, Mean ($\pm$ s.e.) frequency of the nine successive flash trains presented to a female that were followed by her reply; frequencies that differed significantly ($P<0.05$; Wilcoxon matched-pairs signed-rank test, with Holm correction for multiple tests) from the frequency of replies to the mean level are shown solid. b, Mean ($\pm$ s.e.) of the average number of flashes in a female's replies; flash numbers that differed significantly ($P<0.05$) from flash numbers in replies to the character's mean level are shown solid.

Performance medicine

Marcus Jacobson

Charcot: Constructing Neurology. By Christopher G. Goetz, Michel Bonduelle and Toby Gelfand. *Oxford University Press*: 1995. Pp. 392. £40, \$55.

WHY do we need another life of the Parisian neurologist Jean-Martin Charcot (1825–92)? Hasn't the subject been exhausted by the large-scale biography published in French in 1955 (English translation, 1956) by Georges Guillain, who was Charcot's successor at the Salpêtrière, and by three eulogies written in 1925 for the centenary of Charcot's birth by his disciples, Joseph Babinski, Pierre Marie and Achille Souques? They wrote to praise their master but, given their disagreements with him, also to bury him. In this new biography, the authors have resurrected Charcot as a principal constructor of modern neurology and have located him in the broad context of nineteenth-century France.

Once the idol of a few neurologists, Charcot has recently become the common interest of feminists, philosophers of modern culture and art critics, as well as a new breed of historians of modern medicine. Why has the Parisian doctor of medicine become the focus of so much recent attention, whereas his arguably more important contemporaries are neglected by comparison? The reasons for this have rather more to do with Freud, Foucault and feminism than with physiology and pharmacology.

The authors have achieved a detailed and balanced synthesis of many points of view derived from all available sources. The result is neither a hagiography nor a hatchet job. But their work falls short of justifying its subtitle "Constructing Neurology". It establishes Charcot neither as an original thinker, nor as a creator of scientific knowledge, nor as the principal constructor of a new style of clinical neurology. Instead, it suggests an alternative, viewing Charcot as a middle man, a subcontractor, so to say, in the construction of a clinical discipline based on experimental neurophysiology, neuroanatomy and neuropathology. The book shows how he developed his flair as a great communicator and as a successful purveyor of other people's original ideas. But it does not reveal why Charcot failed to make any important scientific discoveries.

Two traditions have historically competed for authority in medicine — call them the bedside versus the benchside traditions. It may be a simplification to see Charcot as only a virtuoso of clinical acumen rather than an advancer of

experimental science. But there is no doubt that he was highly sceptical of the virtues of laboratory methodology. And this was during the time when others, including compatriots such as Claude Bernard (1813–78), were achieving the unification of clinical practice with the basic medical sciences of physiology, pharmacology, biochemistry and genetics. Charcot was vigorously opposed to vivisection, and he underestimated the power of laboratory experiments. So it is

further developed this tradition of diagnosis: his so-called anatomo-clinical method.

But it is a mistake to say, as this book does repeatedly, that Charcot was an empiricist, in the sense that his practice was based on knowledge derived only from experiments. A determinist, perhaps, but not an empiricist in the manner of Broca, Erb, Ferrier and other contemporary neurologists. Erb (1840–1921) was the one who obtained strong evidence for the syphilitic aetiology of what he called tabes dorsalis and what Charcot called locomotor ataxia, a condition that Charcot always insisted was caused by an inherited diathesis, and not by any infectious agent. Charcot also believed in the inheritance of neurasthenia, asocial behaviour, drug addiction and chronic criminality.



"A Clinical Lesson at the Salpêtrière" (1887), by Pierre André Brouillet. Charcot addresses his admiring audience of students, doctors and nonmedical luminaries, keeping them at a comfortable distance.

not astonishing that the names of Magendie (1783–1855) and Bernard are missing from his lectures.

Charcot adopted the method, originated by the great French physicians Corvisart (1755–1821) and Laennec (1781–1826), of correlating signs of illness observed at the bedside with pathological findings at autopsy. This pathological-clinical method had been used successfully before Charcot by other French neurologists, notably Duchenne de Boulogne (1806–75) in his studies of locomotor ataxia, and by Broca (1824–80) in his correlation of brain lesions with disorders of language functions. Between 1862 and 1875 Charcot

His part in constructing neurology was that of an interpreter, of a virtuoso performer, not an original creator. Indeed, chapter five is aptly titled "Charcot and the Artistry of Neurological Practice". The authors make a lot of Charcot's artistry, and whether we applaud depends more on our taste for dramaturgy than on our scientific judgement. We are given a fascinating view of Charcot's theatrical gestures, his rhetorical flourishes, and of his supposed misogyny and reputed exploitation of the vulnerability of his patients — exclusively female — at the Salpêtrière. An important difference between Charcot and his patients was that he knew when he was

acting whereas they did not. Another is that he had the legal power to use them, even to abuse them. Even when his diagnosis might have been close to the point, more often his treatments were not. For example, Charcot himself frequently treated hysteria by cauterization of the cervix uteri. Other surgical operations often used to treat hysteria included the entire gamut of gynaecological assaults, from clitoridectomy and ovariectomy to hysterectomy. Emil Kraepelin (1856–1926) criticized this type of therapy for hysteria as “knife happy”. Charcot did not think of therapy as an experimental science.

He needed an audience but he kept it at a comfortable distance. This is depicted in Pierre André Brouillet's painting “A Clinical Lesson at the Salpêtrière” (1887), which is well discussed here. Charcot faces away from his admiring audience of mostly young men and looks past his half-naked patient. She is about to swoon into the arms of Babinski, whose compassion contrasts with Charcot's seeming indifference: an

apparent lack of empathy that he seems to communicate to his audience. The picture can be interpreted as a study of exhibitionism and voyeurism. It is a lesson in medical ethics as much as in history.

Depictions of Charcot at work in his clinic have continued to bring up questions about medical ethics since they were first raised in the public press of Charcot's time. Is the professor's prestige possible only at the expense of his patients (and perhaps also his students)? Such questions form part of a continuing debate being fuelled by feminists as well as philosophers of science. Perhaps the initiation of that debate about the rights of the patient and the authority of the doctor may be seen to be Charcot's most enduring contribution. In that view, Charcot's legacy to our times is in relation to ethical consciousness rather than to positive scientific knowledge. ▲

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Genes in mind

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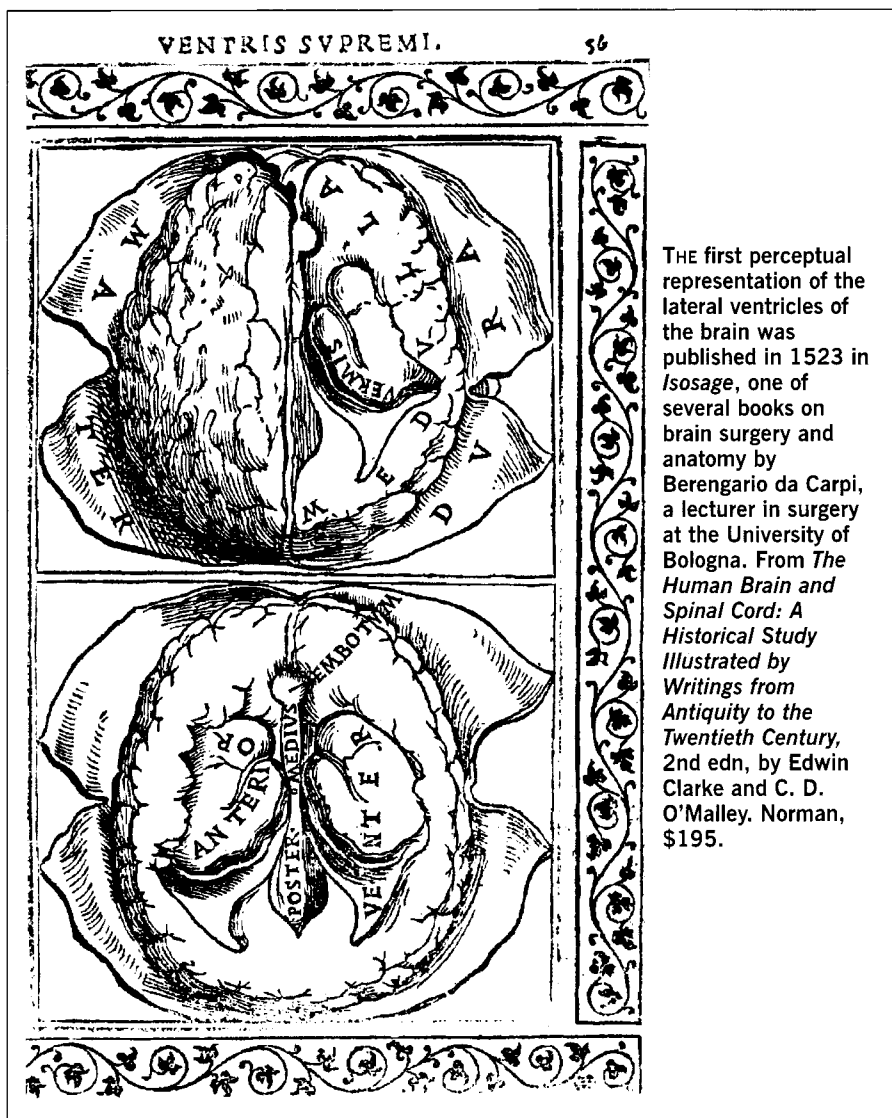
Genetics and Mental Illness: Evolving Issues for Research and Society. Edited by Laura Lee Hall. Plenum: 1996. Pp. 225. \$59.40, £39.60.

THREE powerful yet disparate recent trends in psychiatry and genetics are reflected in this unusual book: the emergence of a new conceptual approach to psychiatric genetics; the rise of mental health advocacy groups; and the burgeoning attention paid to the ethics of genetics research and practice.

Until the mid-1980s, research in psychiatric genetics was dominated by the traditional methods of family, twin and adoption studies, the goal of which was to quantify the familial aggregation of psychiatric disorders and to establish the extent to which such disorders were due to genetic as opposed to environmental factors. In these studies, genes were never directly measured. Rather, their action was inferred from the pattern of resemblance in relatives. By contrast, the past decade has seen an increasing focus on methods that attempt to pinpoint genes conferring susceptibility to psychiatric illness. This shift in emphasis has been driven by advances in molecular genetics in general and in methods of human gene mapping in particular. Although spectacularly successful for Mendelian genetic disorders, and increasingly applied to more complex conditions, these methods have yet to lead to major findings in the study of psychiatric illness. Recent studies hint at a linkage between certain gene markers and schizophrenia, bipolar affective illness and even personality, and may presage more definitive findings in the near future.

The second trend represented in this volume is the rise of consumer advocacy groups for mental health. Traditionally, the mentally ill have fared poorly in the rough and tumble of the political arena where, at least in the United States, decisions are usually made about the funding of both psychiatric research and clinical care for the mentally ill. This situation has, however, begun to change with the emergence of groups made up mostly of the mentally ill and their relatives. These advocacy groups have challenged, in direct and often effective ways, the prejudices with which society continues to view the mentally ill.

The third theme of this book is the ethics of research and practice in human genetics. With the support of funds from the Human Genome Project, the field has addressed a whole range of ethical issues. Few are more difficult than those



confronting psychiatric genetics. It remains to be seen how well the ethical guidelines established for the use of genetic information for breast cancer or Huntington's disease will apply to genes that influence the cognitive distortions and hallucinations of schizophrenia or our vulnerability to alcoholism.

The contrasting perspectives on genetics and mental illness cohabit a bit uneasily in this modest volume. Four of its nine chapters are succinct, well-written reviews by respected researchers. Two of the four are overviews from, respectively, a molecular and a behavioural genetics perspective, and two review current progress on the genetics of schizophrenia and mood disorders.

Juxtaposed with these academic pieces is a moving account of a woman's experiences with obsessive-compulsive disorder. After attending a joint researcher-consumer workshop on psychiatric genetics and feeling the wide gap between the two groups, she identifies the following central dilemma:

If those searching for answers don't know us, or how we really live, or what we think and feel, how likely is what they find, particularly if it's of great importance, to be effectively targeted to reach us? What happens to us while we wait?

Another chapter examines the growing empirical literature on the relationship between creativity and affective illness — posing for the reader the question of how society might cope with a gene implicated in this often devastating disease, which nonetheless may contribute to the genius of a William Blake, a Percy Shelley or a Virginia Woolf.

The policy implications of psychiatric genetics are also explored. Some positive consequences of the research are seen. Genetic findings can lessen the stigma attached to mental illness as well as the parental guilt. Most parents find it more acceptable to conclude that they passed predisposing genes to their offspring than that they caused the illness through inadequate parenting. But it has its negative side, illustrated by a badge (or button to American readers) demanding: "Gene Police! Out of the Pool". To what extent will further discoveries in psychiatric genetics affect the already restricted sexuality and reproductive choices of the mentally ill?

The greatest strength of this book is the variety of perspectives it applies to the questions raised by psychiatric genetics. It will — and should — jar the academic (with his or her mind full of base-pair sequences or statistical models) into seeing the broader picture. Both genes and psychiatric disorders exist in people who struggle to get adequate care, to understand what has happened to them, to search for self-respect and to develop the often limited abilities their illness permits.

But a notable limitation of the volume is its lack of integration across perspectives. Reflecting what is assuredly the current state of the field, from these disparate voices comes only a modest intimation of an emerging dialogue. □

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Whimsy, wit and wordplay

Ian Stewart

The Universe in a Handkerchief: Lewis Carroll's Mathematical Recreations, Games, Puzzles, and Word Plays. By Martin Gardner. Copernicus/Springer: 1996. Pp. 158. £15, \$19.

THERE is no one quite like Lewis Carroll, and no one with quite such an uneven output: from the heights of *Alice* to the depths of *Sylvie and Bruno*, from verse so feeble that one blushes to read it to the immortal *Hunting of the Snark*. He wrote books and articles on mathematical puzzles and logic; he published pamphlets on games he had invented. *The Universe in a Handkerchief* is a package tour of Carroll's whimsy, wit and wordplay. It is a treasure trove of Carrollian oddities: I shall content myself with a selection of typical examples.

Martin Gardner is of course the only possible author, a kindred spirit who even shares Carroll's love of amateur magic.

His title refers to two Carrollian party tricks. In one, mathematical, a handkerchief is sewn to form a projective plane — a one-sided topological surface. In the other, a conjuring trick, the handkerchief is folded to make a jumping mouse: Gardner naturally gives full instructions.

On Christmas Day 1877, Carroll invented a new word-game for "two young ladies having nothing to do", as a *Vanity Fair* booklet states. "Doublets" spread like wildfire and is still popular today. The aim is to turn one given word into another by changing one letter at a time and making valid words at all stages: HEAD HEAL TEAL TELL TALL TAIL. Carroll embedded doublets in instructions such as 'WHY is it better NOT to marry?', adding an extra layer of wit with the answer 'WHY WHO WO WOT NOT'. Doublets are susceptible to mathematical analysis, the most extensive being that of the computer scientist Donald Knuth, who represents the puzzle as the tracing of a graph whose nodes are words, linked by edges when they differ by one letter. Carroll contributed a series of articles on doublets to *Vanity Fair*, collected in the aforementioned booklet in 1879; part of it is included here, along with previously unpublished material hand-lettered by Carroll himself.

In Fit 5 of *Snark*, the Butcher tries to convince the Beaver that $1+2=3$:

Taking Three as the subject to reason about —
A convenient number to state —
We add Seven and Ten, and then multiply out
By One Thousand diminished by Eight.
The result we proceed to divide, as you see,
By Nine Hundred and Ninety and Two:
Then subtract Seventeen, and the answer must be
Exactly and perfectly true.

As Gardner observes, the result of this calculation always reproduces "the subject



Now you see it, now you don't — how to fold and sew a handkerchief to make a closed surface that has no inside or out. Drawn by Harry Furniss for Carroll's *Sylvie and Bruno Concluded*.

to reason about" by virtue of the identity $(x+7+10)(1,000-8)/992-7=x$.

Carroll wrote to newspapers. On 8 April 1856, Jelinger Symons, Her Majesty's Inspector of Schools, wrote to *The Times* to point out that the Moon did not rotate on its axis. (His reason: it presents the same face to the Earth all the time.) Carroll wrote a reply, but it was not among the seven published. Symons persisted; the correspondence continued for a month before petering out. Bizarrely, when the same question was raised ten years later in the United States, the letters column of *Scientific American* debated the issue for three years.

Carroll was a master of deception, leaving innumerable time bombs for later scholars to explode or defuse. A famous letter to Maggie Cunynghame ends with a postscript: "My love to yourself — to your Mother my kindest regards — to your small, fat, impertinent, ignorant brother, my hatred. I think that is all." Although often quoted as prose, it is actually in verse. The lines terminate at 'Mother', 'small', 'brother', 'all'; the metre is perfect. His acrostic dedications are

famous. The prefatory poem to *Alice in Wonderland* uses the word 'little' three times: this is a pun on the three Liddell sisters, one of whom was the real-life Alice.

Carroll couldn't resist puns, good or bad. "How does a doll know that a hand which came off was her right hand?" "Because the other was left." Enjoy. □

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Also of interest

The Red King's Dream: or Lewis Carroll in Wonderland by Jo Elwyn Jones and Francis Gladstone. In a personal quest, prompted by a link between Carroll and their forebear, the Victorian Prime Minister William Gladstone, the authors have revisited Carroll's diaries and haunts, rediscovering little-known information. They have unravelled the code that Carroll used to secrete into his Alice stories his scurrilous views on various figures of Victorian England. Pimlico, £10.

Looking after your invention

Robert I. James

Technology Transfer: Making the Most of Your Intellectual Property. By Neil F. Sullivan. Cambridge University Press: 1995. Pp. 221. £40, \$69.95 (hbk); £14.95, \$24.95 (pbk).

TRANSFERRING technology from academic institutions to industry, especially in the life sciences, is a process that is stimulating much debate. In the United Kingdom, for example, conferences are being held to consider various aspects of technology transfer, the government is setting up initiatives to encourage innovation, universities are building up their technology transfer offices and the Charity Commission is considering how research charities should approach the whole business of commercializing the fruits of their research portfolios.

There are several reasons for this heightened interest. First, transferring technology to a commercial entity (whether a start-up company or an established pharmaceutical company) is the only way for an invention to be developed with a view ultimately to selling the product. In academic medical research directed at discovering new therapies for treating disease, effective technology transfer is therefore the only way that patients worldwide will benefit from new discoveries. Second, successful technology transfer can generate considerable long-term revenues for the inventors, host institutions and

funding bodies. Third, start-up companies based on new technology create jobs and have the potential to bring enormous economic benefits to regions where they are sited.

So this book arrives at just the right time. Aimed mainly at academic researchers in the life sciences, it sets out to discuss all the issues they need to consider to commercialize technology successfully, from deciding whether they have something of value to 'creating a culture' in a new company.

Perhaps the most sophisticated approach to technology transfer in the life sciences is to create a company to exploit a particular piece of technology. Over a period of several years, such companies can bring large returns to their founders as they float on stock markets around the world or form alliances with large pharmaceutical companies. Barely a week goes by without details of new ventures, new rounds of financing or new deals being reported in publications such as *Scrip*, the international pharmaceutical newsletter. But to the author's credit, his book does not just deal with the 'glamorous' subjects but recognizes that technology transfer is an activity that offers a number of possible ways to exploit intellectual property depending on the nature of the technology. Setting up a new company is clearly one of these choices, but other mechanisms are also considered, such as setting up consultancies, contract research

arrangements and collaborative research agreements.

When an academic inventor has made an invention, its potential commercial value will be fundamentally affected by whether or not it is patentable. Patentability is mainly determined by the answer to three questions. Is the invention novel? Did its creation involve an inventive step? Does it have industrial utility? In the first instance, the inventor and his or her advisers will form a view on patentability, but ultimately the issue will be decided by examination of the patent application in the various regional and national patent offices and possibly, if the patent is challenged, in a court of law. The book deals in some detail with the various processes involved in filing and prosecuting a patent application. In addition, it includes a useful section, for scientists working outside the United States, on the importance of maintaining proper notebooks following changes brought about by the General Agreement on Tariffs and Trade Implementation Act in the United States.

As is clear from the later chapters of the book, filing the patent application is only the start of the commercialization process. For the patent application to be a valuable asset rather than an expensive administrative burden, careful consideration must be given to its value and the best way to exploit it. When the commercial strategy has been decided, it needs to be implemented by identifying partners, negotiating agreements, or, if a new company is to be established, attracting investors, and so on. The practicalities surrounding the commercialization of an invention are discussed, with references to further sources of information.

But in dealing with a very wide range of subjects and attempting to describe the various processes in minute detail, the book dilutes the fundamental principles of patenting, valuing and commercializing technology in the life sciences. Nevertheless, at a time when technology transfer is becoming more and more important, any book that tries to pull together a practical overview of the field should be welcomed. □

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New in paperback

The Common But Less Frequent Loon and Other Essays by Keith Stewart Thomson. Yale University Press, £10.95. Essays from the author's "Marginalia" column in *American Scientist*. "Try these essays for bedtime: you can't go far wrong", Michael Taylor, *Nature* **368**, 362 (1994).

Clonal selection and learning in the antibody system

Klaus Rajewsky

Each antibody-producing B cell makes antibodies of unique specificity, reflecting a series of ordered gene rearrangements which must be successfully performed if the cell is to survive. A second selection process occurs during immune responses in which a new antibody repertoire is generated through somatic hypermutation. Here only mutants binding antigen with high affinity survive to become memory cells. Cells expressing autoreactive receptors are counter-selected at both stages. This stringent positive and negative selection allows the generation and diversification of cells while rigorously controlling their specificity.

THE idea that organisms contain specific antibodies before encountering antigen, first conceived by Ehrlich¹, was subsequently dismissed and later revived by Jerne² as the key feature of a specialized immune system, before being developed by Burnet³ and Talmage⁴ into the concept that each antibody-forming cell is genetically committed to express an antibody distinct from that of its companions. The clonal selection theory postulated that the cells express their antibody as a surface receptor and can thus be selected by antigen. Early in development, encountering (self-) antigen leads to cell death, establishing self tolerance. Later, on encountering external antigens, the cells respond by clonal expansion and differentiation into antibody-secreting cells (later called B lymphocytes). This theory explained the induction of specific antibody responses and of self tolerance; the formation of immunological 'memory' by the selective expansion of specialized cells; and immunological 'learning', that is the improvement of antibody quality as the response progresses ('affinity maturation') by selection of cells expressing antibodies with high affinity for antigen. By analogy to bacterial and viral systems, Burnet imagined that affinity maturation would involve selection of rare somatic antibody mutants. Today, these elements of the clonal selection theory still represent the main features of B-cell development revealed by molecular biology.

B-cell and pre-B-cell receptors

One postulate of the clonal selection theory, that B-cell progenitors carry surface antibodies, was soon verified⁵⁻⁷. It took far longer to elucidate how IgM and IgD antibodies (the antibody isotypes first expressed on the B-cell surface in development) could function as signal-transducing receptors, despite their very short cytoplasmic tails. Indeed, some held that surface immunoglobulin (sIg) served merely as an antigen-concentrating device⁸. The solution to this puzzle came through the discovery that sIg expression requires accessory proteins⁹. It is now clear that the B-cell antigen receptor (BCR) is a complex of antigen-binding sIg with the membrane-spanning, signal-transducing heterodimer Ig- α /Ig- β ¹⁰ (Fig. 1). Signal transduction by the BCR is triggered by receptor crosslinking and depends on an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic portions of Ig- α and Ig- β ¹¹, which transmits signals from the BCR to intracellular signalling components. These include tyrosine kinases (such as Syk and the Src family members Fyn, Lyn, Blk and Btk) that act in concert with cytoplasmic and membrane-bound phosphatases^{12,13}.

Immune responses usually involve complex interactions between effector cells and various regulatory cells. Accordingly, B-cell responses depend both on engagement of the BCR by antigen, and on signals through other receptors responsible for

regulation. Some of these receptors are 'co-receptors', which participate nonspecifically in antigen recognition and act together with the BCR (such as the CD19/CD21 complex¹⁴ and the type II Fc γ receptor, which inhibits signalling through the BCR¹⁵). Others mediate the interaction of antigen-activated B cells with helper T cells (see below).

Signals from the BCR and the various co-receptors are integrated inside the cell according to programmes that allow the B cell to react appropriately to environmental stimuli. The elucidation of these programmes is still rapidly advancing and lies beyond the scope of this discussion. Through a combined mutational, biochemical and functional analysis, however, the problem of decision-making in B-cell development is now accessible at the molecular level¹². The power of gene targeting in this area is evident from human diseases in which natural mutations affect B-cell signalling, deficiencies in Btk, CD40 ligand and interleukin-2 (IL-2)-receptor γ -chain corresponding to X-linked agammaglobulinaemia, the hyper-IgM syndrome, and X-linked severe combined immunodeficiency (SCID), respectively¹².

sIg thus functions as an antigen receptor on the B-cell surface, allowing selection by antigen exactly as predicted by the clonal selection theory. Whether an antigenic encounter induces an antibody response or tolerance could either depend on the ontogenetic timing of the encounter or on the presence or absence of a 'second' signal delivered by neighbouring cells (such as T helper cells) through co-receptors on the B-cell surface, as suggested by Bretscher and Cohn¹⁶ (see below).

Cells earlier in the B-cell lineage express the pre-B-cell receptor (pBCR) rather than the BCR (Fig. 1)^{10,17}. Like the BCR, the pBCR contains immunoglobulin heavy (H) chains, but the light (L) chains are replaced by the 'surrogate' L chains $\lambda 5$ and VpreB¹⁸⁻²⁰. Both receptors share Ig- α / β signalling subunits and signal in a similar manner. Such signals, transmitted through the pBCR, play a crucial role in early B-cell development, as discussed below.

Clonal selection of antibody specificity

In his clonal selection theory, Burnet envisaged that a large population of cells, each expressing a distinct antibody specificity, might be generated by genetic 'randomization' of an initial, common specificity. In the chicken, this almost literally describes what happens. In mice and humans, B cells express distinct antibody specificities from the beginning.

In all vertebrates, the genes encoding antibody variable (V) regions are assembled during B-cell development from gene segments termed *V*, *D* and *J* (for the IgH locus) or *V* and *J* (for IgL) through a process of site-specific recombination or 'joining'^{21,22}. Joining involves the introduction of double-strand breaks

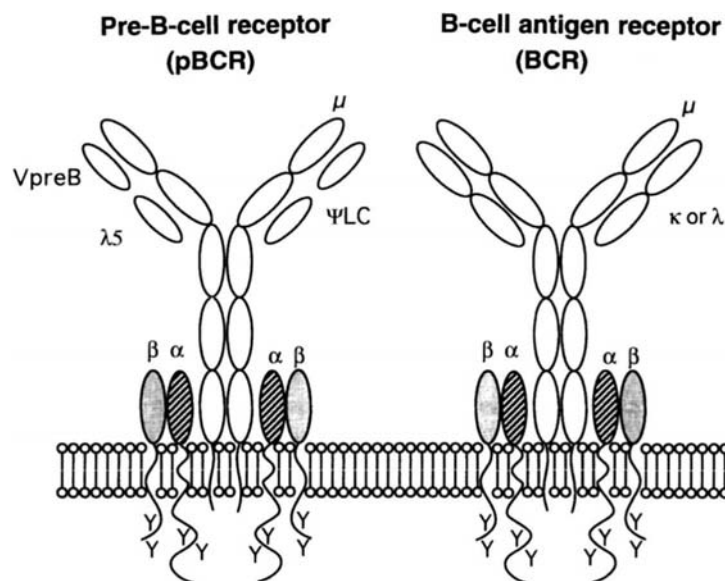


FIG. 1 The B-cell antigen receptor and the pre-B-cell receptor. Y-Y denotes the ITAM motif in the cytoplasmic tails of Ig- α and Ig- β .

at specific recognition signal sequences adjacent to the *V*, *D* and *J* elements by the recombination-activating genes *RAG-1* and -2 (refs 23, 24), followed by double-strand repair²⁵. Whereas the latter process is mediated by general repair enzymes, *RAG-1* and -2 are expressed only at certain stages of lymphoid development.

In the chicken, the same *V*, *D* and *J* genes are assembled in all B-cell progenitors, and all cells initially express the same or similar antibodies. Diversity is generated through subsequent rounds of gene conversion during proliferation, the donor genes being pseudo-*V* genes upstream of the *V* genes that have already been rearranged²⁶. A similar pattern is seen in rabbits, at least in the case of the IgH locus. Here, however, the donor genes are themselves potentially functional, and it is unclear why they are rarely or never used in the initial rearrangements²⁷. In mice and humans, numerous functional *V*, *D* and *J* gene segments are present in both the IgH and the two IgL loci (Ig κ and Ig λ), and each cell joins a different set of segments so that different cells express different receptors from the beginning. Diversity is further increased by variation in the recombination breakpoints and by nucleotide deletions and insertions (so-called N-regions) at those breakpoints, making them by far the most diverse elements in the antibody repertoire²⁸. Other species use different strategies still. Thus in the sheep, diverse *V* and *J* segments are also used, but these rearrangements are further diversified by a mechanism of localized hyper-point-mutation^{29,30}, similar to that operating in antigen-driven antibody responses in mice and humans (see below).

Cell development and allelic exclusion

B-cell progenitors arise early in ontogeny from pluripotent haematopoietic stem cells in a process for which the transcription factors E2A (refs 31 and 32) and EBF (ref. 33) seem to be critical. These cells migrate into specialized cellular microenvironments where they are clonally selected. In the chicken, this happens in the bursa of Fabricius, which contains roughly 2×10^9 rapidly dividing B lymphocytes³⁴. In the sheep, the main sites responsible appear to be the ileal Peyer's patches, large organs harbouring billions of rapidly dividing B-lineage cells³⁵. In both species, B-cell generation is largely restricted to a few weeks or months of postnatal life. In mice and humans, B-cell generation begins in embryonic life in the para-aortic tissues, liver and spleen. After birth, it is largely restricted to the bone marrow, where it continues at a diminishing rate throughout life³⁶.

For cellular selection in the sense of Burnet's theory, each B lymphocyte should ideally express only one *V_H*- and one *V_L*-region gene. This principle is called allelic exclusion, or, in the case of κ

and λ L chains, isotype exclusion. Although somatic gene rearrangements produce a unique *V*-region gene at a given IgH or IgL locus, additional control mechanisms are required to guarantee allelic or isotype exclusion, and these again differ in different species.

The case of the chicken

In the chicken, the initial IgH and IgL rearrangements have occurred before the progenitor cells enter the bursa of Fabricius. These rearrangements are apparently independent of each other, and usually only one of the two IgH and IgL alleles is rearranged²⁶ (chickens appear to possess only one, λ -like, IgL locus). It is unclear how this restriction is achieved. One possibility is that only a few rearrangements can occur within the time window in which rearrangements are permitted, perhaps as a result of a silencer of rearrangement²⁶, restricting the number of progenitor cells reaching the bursa of Fabricius. This may be why the cells massively expand in the bursa, with concomitant antibody diversification.

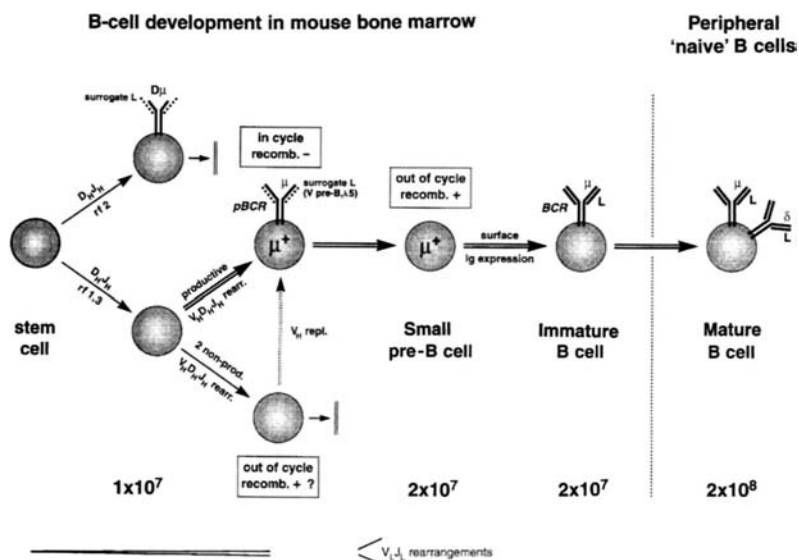
Monospecific B cells in mice and humans

In mice and humans, the gene rearrangements allow efficient generation of allelically excluded cells with diverse receptor repertoires from the beginning.

The main elements of this ingenious mechanism were first postulated by Alt and Baltimore in their 'ordered' model, based on studies using pre-B-cell lines transformed with Abelson virus³⁷. They suggested that gene rearrangements are initiated at the IgH locus, with *D_H* $\rightarrow$ *J_H* followed by *V_H* $\rightarrow$ *D_HJ_H* rearrangement. If a *V_HD_HJ_H* joint is 'productive' (that is, in-frame), a heavy-chain of class μ is expressed from this joint, producing a pre-B cell. Earlier work had suggested that such pre-B-cells³⁸ represented a major fraction of B-cell progenitors in the bone marrow^{39,40}. The ordered model postulated that μ -chain expression was critical for further development, triggering the termination of rearrangements at the IgH locus (ensuring allelic exclusion) and the initiation of rearrangements at the L-chain loci. Here again, an order of rearrangements (Ig κ before Ig λ) was postulated. On acquiring a productive *V_LJ_L* joint—either *V κ J κ* or *V λ J λ* —the cell would express a light chain in addition to its heavy chain (that is, a complete antibody molecule), and rearrangements would be terminated altogether.

A key feature of this model was that the productive rearrangements at the immunoglobulin loci should be 'sensed' by the cell by means of L- and/or H-chain expression. This became more plausible with the discovery that B-cell progenitors can express membrane-bound μ chains without κ or λ chains⁴¹ as a component

FIG. 2 Scheme of B-cell development in the bone marrow of the mouse. The two major checkpoints in this pathway, marked by pBCR and BCR expression respectively, are indicated. A third checkpoint at the stage of D_H-J_H joining, marked by expression of a truncated μ -chain (D_μ protein) from a subset of D_HJ_H joints (characterized by one of the three D_H reading frames (rf)) is also shown. Cells carrying non-productive $V_HD_HJ_H$ joints on both IgH loci may be rescued by V_H gene recombination into the $V_HD_HJ_H$ joint (V_H replacement). The evidence that these cells are out of cell cycle but can possibly continue gene recombination (recomb. +; unlike early progenitor cells carrying productive $V_HD_HJ_H$ joints), is based on the analysis of gene rearrangements and expression of PCNA (ref. 176) and *RAG-1* genes in single, flow-cytometrically isolated progenitor cells (A. Ehlich, unpublished data). pBCR expression may draw the cells into cycle and is rapidly downregulated while the cells are still in cycle. (For the puzzling difficulty to detect surface expression of pBCR see refs. 17 and 182) The generation of immature B cells from small pre-B cells and their entry into the peripheral B-cell pool occurs in the absence of cell proliferation. Approximate cell numbers are given for the total bone marrow of the mouse (the first number of the left including cycling, large pre-B cells). For further references and explanations, see text.



of the pBCR (ref. 17). Expression of pBCR and of BCR may thus mark the two checkpoints in B-cell development postulated in the ordered model.

Analysis of the order and interdependence of rearrangements in normal B-cell development required new technical tools, including the separation by flow cytometry of B-cell progenitors at different stages of maturation using cell-surface markers⁴²; the growth of these progenitors in cell culture, using the growth factor IL-7 and feeder layers of stromal cells⁴³⁻⁴⁵; the targeted mutagenesis of the mouse germ line⁴⁶; and the amplification of gene rearrangements from small populations of, or even single⁴⁷ progenitor cells. Using these techniques, it became clear that in mouse B-cell progenitors, both *in vitro*¹⁷ and *in vivo*^{42,47,48}, gene rearrangements usually begin at the IgH locus, with $D_H \rightarrow J_H$ preceding $V_H \rightarrow D_HJ_H$ and both IgH alleles undergoing $D_H \rightarrow J_H$ joining. In agreement with data from B-progenitor cell lines in both mice^{17,49} and humans⁵⁰, however, rearrangements of Igk are not entirely dependent upon the expression of membrane-bound μ -chains, the pBCR, or indeed any rearrangement of the IgH locus, because they are seen at low frequency in progenitor cells from mouse mutants deficient in these properties^{48,51,52}.

Similarly, most murine progenitor cells begin their L-chain rearrangements at Igk, but Igk rearrangement is not required for Ig λ rearrangement as the latter occurs at increased efficiency in mutants unable to perform the former⁵³. In addition, a small fraction of progenitor cells in wild-type mice rearrange Ig λ before Igk⁵⁴. While establishing an overall 'order' of immunoglobulin gene rearrangements *in vivo*, these results collectively suggest that rearrangements at different loci occur at intrinsically or competitively different frequencies, which may vary over developmental time. This does not exclude upregulation of L-chain rearrangements by expression of membrane-bound μ -chains or the pBCR, as suggested by experiments in pre-B cells transformed by Abelson virus⁵⁵⁻⁵⁷.

Checkpoints in early B-cell development

The central issues of the ordered model are whether μ -chain (or pBCR) expression early in B-cell development is required for further development, and whether it signals allelic exclusion at the IgH locus. The first question was directly addressed by inactivating the membrane exon of the μ -chain gene in the mouse germ line⁵¹. B-cell development was arrested at an early stage, before the cells entered the pool of resting, small pre-B cells^{48,51}. The same phenotype was later seen in mouse mutants unable to rearrange the IgH (refs 48 and 52) or any immunoglobulin locus^{58,59}, and, to

a lesser extent, in mice deficient for $\lambda 5$ (refs 48 and 60), which is required for pBCR expression (Fig. 1). In rearrangement-deficient mice (including also the SCID mutant⁶¹, which is deficient in repairing DNA double-strand breaks), the block in B-cell development could be overcome by introducing an H-chain transgene (allowing pre-B-cell development) or H- and L-chain transgenes (allowing efficient generation of monoclonal B cells)⁶²⁻⁶⁴. The finding that cells that carry non-productive $V_HD_HJ_H$ joints on both IgH alleles accumulate at a developmental stage preceding that of μ -chain-expressing pre-B cells⁴⁷ showed that these results were relevant to normal B-cell development.

Expression of μ -chains, presumably in the pBCR complex, is thus essential for early B-cell development. The majority of the cells (60–85%) beginning to express μ -chains and pBCR are in the S or G2 phase of the cell cycle⁶⁵, a higher fraction than at any other stage of early B-cell development. pBCR expression thus appears to trigger proliferation of μ -chain-expressing cells, although the extent of proliferation is controversial, the estimates ranging from a single to six divisions^{66,177}.

Experimental evidence also supports a role of membrane μ -chain or pBCR expression in signalling allelic exclusion at the IgH locus. Transgenes encoding an intact μ -chain^{67,68} (specifically, its membrane-bound form^{69,70}) prevent or profoundly impede rearrangements at the endogenous IgH loci. Conversely, mutants expressing only the secreted form of the μ -chain from one chromosome generate allelically 'included', double-producing cells⁷¹. Furthermore, D_μ protein, a truncated, membrane-bound μ -chain expressed in the mouse from a subset of V_HJ_H rearrangements⁷² (Fig. 2), like the membrane-bound μ -chain, appears to inhibit $V_H \rightarrow D_HJ_H$ joining^{47,73,74}, preventing further development of D_μ -expressing progenitor cells. Indeed, D_μ protein can be expressed on the surface of pre-B cell lines in a complex resembling the pBCR⁷⁵. Perhaps the most direct demonstration of pBCR-mediated allelic exclusion comes from the analysis of $V_HD_HJ_H$ rearrangements in single progenitor cells from wild-type and $\lambda 5$ -deficient mice. In the former, allelic exclusion is seen from the onset of $V_H \rightarrow D_HJ_H$ joining, whereas the latter are allelically 'included'⁷⁴.

How pBCR expression mediates allelic exclusion is still unclear. Recent data indicate that the cytoplasmic domains of Ig- α and/or Ig- β are involved⁷⁶ and that pBCR expression is correlated with *RAG* gene down-regulation⁷⁷. The latter, however, can only partly explain allelic exclusion, because the *RAG* genes are later expressed again to allow rearrangements at the IgL loci, without inducing further IgH rearrangement.

BCR expression on newly generated B cells marks the second 'checkpoint' in B-cell development: B-lineage cells devoid of sIg are hardly seen in the peripheral immune system. In small pre-B and immature B cells, the survival gene *bcl-2* is down-regulated^{78,79}, consistent with the notion that these cells are programmed to die unless rescued into the long-lived peripheral B-cell pool (where *bcl-2* is again upregulated⁷⁸) by a hypothetical BCR-mediated signal. The existence of such a signal is in line with the demonstration that truncation of the cytoplasmic domain of $\text{Ig}\alpha$ (Fig. 1) reduces the peripheral B-cell pool 100-fold, whereas the population of immature B cells in the bone marrow is reduced only 4-fold⁷⁸. The cells in the peripheral B-cell pool appear thus selected for carrying a functional BCR on their surface, and not just membrane-bound antibody.

Collectively, these results support the scheme of B-cell development depicted in Fig. 2. Early in development, immunoglobulin gene rearrangements are initiated predominantly, but not exclusively, at the IgH loci. Cellular maturation is independent of these rearrangements until the expression of membrane-bound μ -chains—presumably forming pBCR—becomes critical. Cells unable to express μ -chain are blocked in development and eventually die, although they persist for some time⁴⁷. Cells expressing the pBCR terminate $V_H \rightarrow D_HJ_H$ rearrangement, expand through an unknown number of cell cycles, down-regulate surrogate L-chain expression^{77,79} and become small, resting pre-B cells. Immature B cells are generated from this population by upregulation of L-chain gene rearrangement. These in turn may become members of the peripheral pool of long-lived mature B cells, provided they express a functional BCR.

Efficiency of B-cell generation

The scheme depicted in Fig. 2 involves proliferative expansion of the progenitor population. From the time the B-cell-lineage marker CD45R/B220 is expressed, close to the onset of gene rearrangement, the cells appear to undergo 5–6 divisions, most of which seem to precede μ -chain expression⁸⁰. However, at the checkpoint of μ -chain (or pBCR) expression most cells are in cycle, and by analogy with thymic T-cell development⁸¹, might expand significantly also at that stage (see above). Depending on the extent of expansion, a given μ -chain could be expressed in the emerging B cell population in combination with several different L chains.

Rearrangement in the progenitor cells must be accompanied by substantial cell loss at the checkpoint of pBCR expression. Assuming that $D\mu$ -expressing cells are completely eliminated and that 20% of the $V_H D_H J_H$ rearrangements in the remaining cells are productive⁷³, 56% of the cells will be lost at $D_H J_H$ joining and 64% at membrane μ -chain expression, or 84% altogether, in good agreement with Osmond's earlier estimates⁸⁰. (These are upper estimates as some cells may be rescued by secondary $D_H J_H$ rearrangements or by so-called V_H gene replacement, that is, the rearrangement of an upstream V_H gene into a $V_H D_H J_H$ complex^{82,83}.) This calculation only holds for the adult, however, where N sequences are added to most $D_H J_H$ joints^{84,85}. In the newborn, N addition is rare, and by the rule of 'homology joining'⁸⁴, $D_H J_H$ joining in one of the three D_H reading frames is favoured. This reading frame prevents $D\mu$ protein expression and is essentially devoid of stop codons⁷³. Thus, virtually no cells are lost at the stage of $D_H J_H$ joining early in ontogeny, and the fraction of productive $V_H D_H J_H$ joints rises to over 30%, reducing losses at the checkpoint of membrane μ expression to less than 50%. The efficiency of pre-B-cell generation is thus increased while the immune system is built up, but at the cost of a restricted receptor repertoire.

Unlike pre-B-cell production, the pre-B to B-cell transition may involve little cell loss, as successive gene rearrangements at the $\text{Ig}\kappa$ locus (involving unrearranged upstream $V\kappa$ and downstream $J\kappa$ elements) are possible and do indeed occur^{86,87}. Moreover, the $\text{Ig}\lambda$ loci are also available, where again multiple rearrangements are at least formally possible. This unique flexibility not only enhances

the efficiency of B-cell generation from pre-B cells, but also allows an 'immature' B cell to exchange one productive $V_L J_L$ rearrangement for another (see below).

Loss of newly generated B cells (expressing IgM but not yet IgD antibodies on their surface) *en route* to the peripheral pool of $\text{IgM}^+ \text{IgD}^-$ mature B cells nevertheless appears to be dramatic. The daily production of immature B cells in the mouse is about 10–20 million⁸⁰. Of these, only 10% appear to reach the periphery, and 70% of the emigrants seem to die after a few days⁸⁸. Only 3% of the immature B cells generated in the bone marrow thus become mature, long-lived B cells, with life spans of weeks or months⁸⁹, and in humans perhaps even decades⁹⁰. As discussed below, this loss at least partly reflects selection on the basis of receptor specificity. This is one reason why the receptor repertoire expressed in the peripheral pool of $\text{IgM}^+ \text{IgD}^-$ B cells differs from that expressed in the bone marrow population of pre-B and immature B cells²².

Selection of the primary antibody repertoire

The mechanisms shaping the primary antibody repertoire (that is, the repertoire expressed by the cells joining the peripheral pool of $\text{IgM}^+ \text{IgD}^+$ cells) fall into two categories. First, the mechanisms of antibody diversification can themselves mediate selection. In the newborn mouse, the repertoire is restricted by the paucity of N-region insertion^{84,85,91}, together with the preferential recombination of V , D and J elements at short sequence homologies^{84,92}. Later, diversity is drastically increased by N-region insertion at the borders of D_H and, in humans, the $V\kappa$ – $J\kappa$ border^{84,85,91,93–95}. Diversity is also restricted by the inability of certain H and L chains to pair properly^{96,97} and by differences in the usage of the various germline V , D and J elements⁹². Although such mechanisms can have impressive effects, such as the predominant expression of the $V8IX V_H$ gene early in mouse ontogeny²², their physiological meaning remains enigmatic. Indeed, targeted deletion of the $V8IX$ gene in mice has little effect on B-cell development (F. Huetz, D. Holmberg, A. Coutinho and K.R., unpublished data).

Second, there is ligand-mediated negative selection of cells expressing autoreactive antibodies, exactly as postulated in the clonal selection hypothesis⁹⁸. Transgenic mice, in which most newly generated B cells express an antibody against an antigen with strong crosslinking capacity that is present in the bone marrow, have no such B cells in their peripheral immune system^{99–102}. This negative selection apparently reflects either apoptotic death on encountering antigen (as seen in B-cell lines *in vitro*¹⁰³ and in mature B cells lacking T-cell help *in vivo*¹⁰⁴) or rescue of the autoreactive cells by further immunoglobulin rearrangement. Depending on their affinity for antigen, autoreactive cells may survive in the bone marrow for long enough^{105,106} to be rescued if their receptor is replaced by an 'innocent' one^{97,107,108}. Such receptor editing is most easily imagined at the $\text{Ig}\kappa$ locus, which can undergo successive rearrangements^{86,87} and can be inactivated by so-called RS recombination²², facilitating $\text{Ig}\lambda$ rearrangement. Shifts from κ to λ chain expression have indeed been observed in immature mouse and human B cells *in vitro*¹⁰⁹.

The original concept of receptor editing involved *RAG* gene reactivation triggered by interaction of the BCR with antigen¹⁰⁷, inducing immunoglobulin gene rearrangements in immature B cells. The evidence for this is indirect^{107,108}, however, and the issue remains controversial, particularly as it is unclear what initially terminates *RAG* gene expression in newly-developing B cells.

Newly generated autoreactive B cells are also negatively selected on encountering soluble (monomeric?) antigen, producing inefficient BCR crosslinking. Such cells can leave the bone marrow, but down-regulate surface IgM and enter an anergic (antigen-unresponsive) state¹¹⁰. Anergy can be similarly induced in mature B cells¹¹¹. Cell transfer experiments have shown that the anergic cells fail to compete with normal cells in populating peripheral lymphoid organs and may be counter-selected on reaching the spleen before entering B cell follicles—a possible further checkpoint in B-cell development¹¹².

It remains to be shown to what extent the efficient elimination of even weakly¹¹³ autoreactive B cells in the transgenic systems (in which the transgenic antibodies may often be prematurely expressed) can be extrapolated to normal physiology. Autoreactive antibodies indeed occur in the primary antibody repertoire, and may even be positively selected by antibody V region (that is, idiotypic) interactions^{114,179}. Such processes seem to be restricted to early ontogeny and perhaps a particular B-cell subset, the so-called B-1 cells (see below). Their physiological impact remains obscure. Other autoreactive receptors may simply escape deletion, if insufficient antigen is available in the bone marrow. The censorship of newly generated B cells necessarily involves positive as well as negative selection, however, in that only B cells expressing a functional BCR reach the periphery. Whether this positive selection is based on cell-autonomous mechanisms or mediated by ligands in the microenvironment is still unclear.

In principle, the primary antibody repertoire could also be shaped by ligand-mediated selection of pBCR-expressing cells on the basis of their V_H regions. While pBCR expression (like BCR expression) certainly results in positive selection, and cells expressing certain μ chains may do better than others at that stage¹¹⁵, the experimental evidence for this intriguing idea is again only indirect¹¹⁶.

The antibody response

In mice, the peripheral B-cell pool consists of a few hundred million cells. More than three quarters of these are mature, IgM⁺ IgD⁺ B cells, the main source of cells involved in primary immune responses. The remaining B cells form several smaller subsets, including memory cells (discussed further below) and the so-called B-1 cells¹¹⁷. The latter, characterized by cell-surface markers like CD5 and Mac-1 (ref. 118), dominate the B-cell population early in ontogeny, but are later largely confined to the peritoneal and pleural cavities, where they participate in local gut- and lung-associated immune responses. Although the origin of these interesting cells is still controversial, there is some evidence that they may represent an ancient, separate cell lineage¹¹⁹ involved in 'natural' defence against common pathogens in the environment^{119,120}. Like B cells in chicken and sheep, B-1 cells appear mainly to be generated early in ontogeny¹¹⁸. They then propagate themselves throughout life, supposedly driven by self- and environmental antigens. Certain antibody specificities, some of which recognize bacterial and (cross-reacting?) self-antigens, are indeed strongly selected in B-1 cells, and maintenance of the cells seems to depend on the co-receptor CD19, which has a critical role in certain forms of antigen-driven B-cell selection^{121,122}. In accord with their presumed role in natural defence, B-1 cells appear to be major contributors to 'natural' antibody production¹²³ (that is, the production of antibodies without intentional immunization or exposure to any environmental antigen¹²⁴).

Induction of antibody responses in B-1 and conventional B cells requires presentation of antigen in an immunogenic form. Multimeric antigens, particularly on cell surfaces, can often directly stimulate B-cell proliferation and differentiation into antibody-secreting plasma cells by efficient crosslinking of the BCR¹²⁵. Both conventional and B-1 cells can participate in these T-cell-independent responses¹²³, which produce mainly IgM and IgG3 antibodies.

By contrast, T-cell-dependent responses¹²⁶ are elicited by protein antigens that cannot trigger an antibody response by themselves. In this case, from the B-cell's perspective, antigen is first complexed by natural antibody and components of the complement system, and is then presented to B cells in complexed form on antigen-presenting cells, taken up by the B cell through its BCR (a function of the BCR independent of the ITAM motif; ref. 127), and fragmented into peptides inside the cell (processing). The resulting peptides are then presented by class II molecules of the major histocompatibility complex (MHC) to T helper cells, which recognize the peptide-MHC complex by means of the T-cell

receptor and its CD4 co-receptor, triggering the B-cell response.

Functional interaction between B cells and the other cells involved again requires that the cells also interact through co-receptors and their ligands. Thus, B-cell activation by antigen on follicular dendritic cells (FDC) involves both the BCR and the co-receptor CD19 (ref. 14). Similarly, CD80 and CD86 are upregulated by BCR crosslinking, binding to CD28 and CTLA-4 on the T-cell surface and stimulating helper-cell activity, whereas further activation of the B cell depends on its interaction through CD40 with CD40 ligand on the T-cell surface. T-cell-dependent responses generally, although not always¹²⁸, involve conventional B cells. It is in these responses that such cells generate immunological memory and a new antibody repertoire by somatic hypermutation of their antibody genes.

Secondary antibody repertoire

Affinity maturation of antibodies¹²⁹⁻¹³¹ and immunological memory are classical phenomena. Both reflect a process of somatic evolution in which mutants are generated from the primary antibody repertoire as first demonstrated by Weigert, Cohn and coworkers in murine λ chains by amino-acid sequencing¹³², and selected on the basis of improved affinity for, or on-rate of binding to¹³³, the antigen. This astonishing process goes beyond Burnet's view, in that the antigen not only selects rare antibody mutants on the basis of their affinity, but also induces their generation (as advocated many years ago¹³⁴), triggering their hypermutation in antigen-activated B cells in a special pathway of differentiation, the germinal centre response. It thus focuses on those B cells already expressing antigen-specific antibodies, albeit usually of low affinity—an ingenious way to initiate efficient affinity maturation.

Affinity maturation

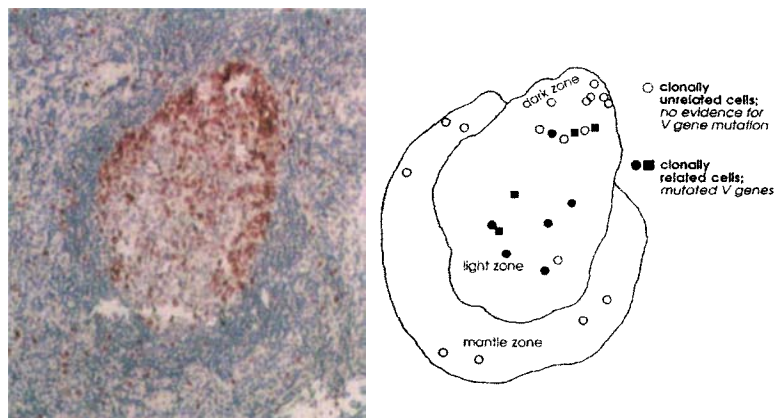
Assessing the contribution of somatic mutation to antibody diversity required a comparison of antibody V-region genes in the germ line with those carried by B cells at various stages of differentiation. This was mainly achieved by analysing antibody responses restricted to one or a few V_H and V_L elements, initially by examining antibody-secreting hybridomas isolated from various stages of the response¹²⁰, and later by amplifying rearranged V-region genes from cell populations or isolated single cells¹³⁵⁻¹³⁹. A clear pattern emerged: the antibodies were virtually unmutated early in the response, but point mutations were seen in the V (but not C) regions at increasing frequency as the response progressed^{140,141}. In the secondary response, most antibodies were highly mutated, with mutation frequencies in the per cent range¹²⁰. Both silent and replacement mutations occurred, and *in vitro* mutagenesis experiments showed that in the response to a given antigen, 'key' mutations repeatedly found in the mutated antibodies indeed increased the antibody's affinity^{141,142}. Moreover, the existence of sequences sharing identical, unique $V_H D_H J_H$ or $V_L J_L$ rearrangements, but differing by point mutations, allowed the construction of genealogical trees, indicating that somatic mutation occurred stepwise in the course of cell proliferation¹⁴³, corresponding to stepwise affinity maturation¹⁴⁴.

The mechanism responsible for mutation is targeted to rearranged V-region genes¹⁴⁵ and their immediate vicinity¹⁴⁶, introducing point mutations at a rate so high (about 10^{-4} – 10^{-3} per base pair per generation¹⁴¹⁻¹⁴³) that irrelevant (for instance, silent) mutations are co-selected with mutations that increase affinity. The process of mutant generation and selection must thus be accompanied by substantial cell loss.

Somatic hypermutation

The pattern of somatic mutation is generally far from random. Replacement (R) mutations are often overrepresented in complementarity-determining regions (CDRs) and underrepresented in the framework regions, whereas the opposite is true for silent (S) mutations. This bias has been taken as evidence for antigenic selection of the mutants¹²⁰. Although such selection certainly occurs, the mere determination of R/S ratios can be

FIG. 3 Histological section of a germinal centre (GC) from a human tonsil. On the left is the section as it appears under the microscope after staining with haematoxylin (blue) and the antibody Ki67, which recognizes an epitope in proliferating cells (red). The GC cells react with this antibody, most intensely in the area of most rapid proliferation, the so-called dark zone. The GC is surrounded by a 'mantle' of naive, resting B cells (blue), a remnant of the B-cell follicle in which the GC arose¹⁵⁷. It is believed that somatic hypermutation is set in motion in the dark zone of the GC, and high-affinity mutants are selected in the 'light' zone¹⁵⁷. This model is supported by the sequence analysis of Ig gene rearrangements amplified by polymerase chain reaction (PCR) from single cells picked by micromanipulation from the GC on the left. This revealed polyclonal B-cell proliferation at the base of the dark zone and expansion of two large B-cell clones into the GC, with ongoing somatic mutation during the course of proliferation (right). Taken from ref. 160, with modifications.



misleading, because the mechanism introducing the mutations has strong preferences for certain sequences, like any other such mechanism. Certain bases are thus preferentially replaced by certain others, and pronounced hotspots of hypermutation exist^{30,146,147,148}. Intriguingly, the V-region gene sequences have apparently evolved to match the biases of the hypermutation mechanism, so that mutations preferentially accumulate in CDRs and biased R/S ratios are seen even without antigenic selection^{30,149,150}. This adaptation may be critical in reducing the load of useless mutants and thus optimizing the affinity maturation process.

The nature of the 'mutator' is still enigmatic (some form of error-prone DNA repair being a favourite speculation now and in the past¹⁵¹), but dramatic progress has been made in defining the *cis*-acting elements that target the mutations to rearranged V-region genes, irrespective of whether the rearrangements are in or out of frame. Using κ -chain transgenes that undergo somatic hypermutation to the same extent as the endogenous V-region genes *in vitro*, it has been demonstrated that the intron- and 3'-enhancers in the murine Igk locus are critical for $V\kappa J\kappa$ hypermutation, whereas the target of hypermutation can apparently be any sequence in the position of the $V\kappa J\kappa$ gene and the $V\kappa$ promoter can be exchanged for another promoter^{152,153}. Recent work¹⁵⁴ indicates that transcription initiation may be involved in targeting hypermutation. Given the rapid progress in this area and that of DNA repair, as well as the availability of hypermutating cell lines^{155,156}, the molecular mechanism of somatic hypermutation may soon be elucidated.

The germinal centre reaction

Affinity maturation and memory generation have long been thought to take place in the special microenvironment of germinal centres¹⁵⁷ (Fig. 3). Germinal centres arise inside 'follicles' composed of naive B cells on immunization with T-cell-dependent antigens. They are characterized by the rapid expansion of an oligoclonal population of antigen-activated B cells in a highly organized microenvironment whose main other components are antigen-specific helper T cells^{158,159} and FDC. The FDC carry antigen complexed to antibodies and components of the complement system on their surface, and this form of antigen presentation, together with signals delivered by the T helper cells, is thought to be critical for the selection and maturation of high-affinity memory cells¹⁵⁷.

Evidence that somatic hypermutation indeed takes place in germinal centres was obtained by amplifying and sequencing V-region genes from populations of purified germinal centre B cells¹³⁶ or from cell populations¹³⁵ or single cells¹⁶⁰ picked from individual germinal centres on histological sections (Fig. 3). The latter experiments showed directly that a few B-cell clones initially grow out from a polyclonal population drawn into the germinal centre and populate most of it while rapidly accumulating somatic mutations¹⁶⁰. By contrast, follicular B cells surrounding the germ-

inal centre, like early antibody-secreting cells, express unmutated antibodies^{135,160}.

Producing antibody mutants on a large scale and at a high rate calls for stringent selection mechanisms guaranteeing that only high-affinity mutants survive, and eliminating low-affinity or autoreactive mutants. The isolation of mutant genes from the total B-cell population at various times after immunization showed that stringent selection indeed occurs. Mutants accumulated rapidly over the first few weeks (matching the kinetics of the germinal centre response), and from early on a key mutation conferring high affinity (and in this instance not representing a mutational hotspot) was strongly selected in the population¹³⁸.

Unlike resting, naive and memory B cells¹⁶¹, proliferating germinal centre cells express only low levels of Bcl-2 (but high levels of CD95/Fas) and seem programmed to die (as suggested by *in vitro* experiments) unless rescued by signals involving antigen and antigen-specific T cells¹⁵⁷. Cells expressing high-affinity antibodies can thus be positively selected in the microenvironment of the germinal centre, and competition between mutants for antigen and T-cell help may be an important contributor to efficient selection.

Another such factor may be the isotype switch which takes place in the germinal centre¹⁶². This is mediated by non-homologous, site-specific recombination and leads to the expression of antibodies of class G, A or E, instead of the original classes M and D (ref. 163). The cytoplasmic tails of the corresponding H chains are longer than those of μ and δ (28, 14 and 28 amino acids, respectively, as opposed to three) and are well conserved, but the impact of their expression on BCR signalling remains to be explored.

In striking analogy to T-cell selection in the thymus, germinal centre B cells can also be negatively selected by ligands binding to their BCR. They undergo rapid apoptosis on encountering antigen in the absence of T-cell help¹⁶⁴⁻¹⁶⁷, a mechanism that may further minimize the risk of autoreactive mutants.

After a few weeks, the germinal centres shrink and largely disappear. Surviving B cells, which have probably undergone several rounds of positive selection in the germinal centre, upregulate *bcl-2* (ref. 161) and enter a resting state¹⁶⁸. These cells express a novel repertoire of hypermutated, high-affinity antibodies.

Memory cells and secondary response

In mice and humans, memory B cells form a minor subset of B cells and can be distinguished by their somatically mutated antibodies, lack of IgD and (in humans) characteristic surface markers. They are mostly resting, long-lived and in part recirculating cells, many but not all of which have switched isotype from IgM to IgG, A or E^{95,137,161,168,180,181}. Whether the lifespan of these cells can be influenced by occasional contact with persisting antigen is debatable¹⁶⁹, as an accurate measurement of B-cell

memory *in vivo* would require an environment free of persisting antigen, a condition not yet satisfactorily met.

Recent evidence suggests that memory B cells may home preferentially to mucosal surfaces¹⁷⁰, where they can rapidly respond to incoming pathogens. Memory B cells present antigen very efficiently to T cells^{170,180}, so that renewed antigenic contact rapidly draws both T and B memory cells into the secondary response. In this response, proliferative expansion can take place without further hypermutation¹⁷¹, and the cells finally undergo terminal differentiation into antibody-secreting plasma cells. The lack of somatic mutation at this stage may simply reflect the fact that most cells proliferate outside the germinal centre¹⁷². Some cells, however, may re-enter the germinal centre pathway and go through further rounds of mutation and selection¹⁷³, further propagating and optimizing B-cell memory.

The overall picture

Both naive B cells and somatically mutated memory cells are produced by a process involving cell proliferation accompanied by stringent cellular selection, so that only cells of the desired phenotype survive.

In both processes there is positive selection, first of cells with an in-frame $V_H D_H J_H$ rearrangement, which can thus express a μ -chain in the pBCR; then of B cells expressing a complete antibody in a functional receptor complex, presumably through expression of the BCR; and finally of somatic mutants expressing high-affinity antibodies in their BCR in the germinal centre reaction. Only in the last case do we know the ligand that drives the selection (antigen); at earlier stages, selection may be driven by ligands on cells in the environment or by cell-autonomous mechanisms. In all cases, however, unselected cells are rapidly lost from the system, presumably by apoptosis¹⁷⁴.

Both processes also employ antigen-mediated negative selection to avoid self-reactivity. In the germinal centre, the distinction between positive and negative selection may lie in the presence of absence of T-cell help. In the case of B-cell generation, this problem remains to be resolved.

It seems to be a general principle in multicellular organisms that cells unable to respond appropriately to environmental signals die by apoptosis¹⁷⁵. The positive and negative mechanisms for selecting B cells that are discussed here (operating, with some variation, in T cells as well⁸¹) directly reflect this principle. They play a dominant role in the immune system because of the continuous generation and diversification of cells whose receptor repertoire needs rigorous control. Following proliferative expansion in bone marrow and germinal centres, during which the cells are programmed to die if they cannot pass the various control checkpoints, the cells enter a resting state and become long-lived naive B cells or memory cells, ready to perform the functions for which they have been selected. □

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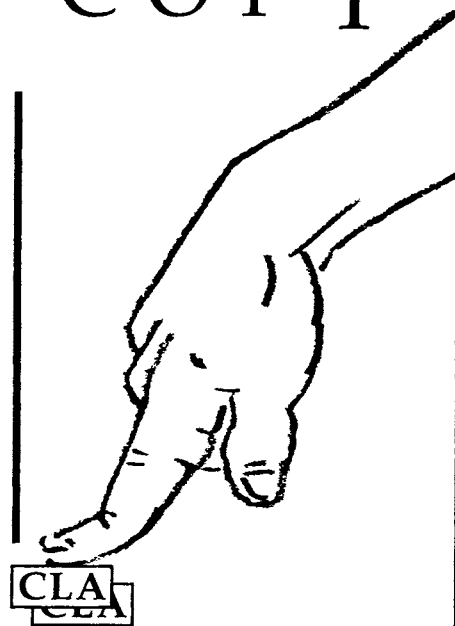
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Star-forming galaxies at very high redshifts

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Analysis of the deepest available images of the sky, obtained by the Hubble Space Telescope, reveals a large number of candidate high-redshift galaxies. A catalogue of 1,683 objects is presented, with estimated redshifts ranging from $z = 0$ to $z > 6$. The high-redshift objects are interpreted as regions of star formation associated with the progenitors of present-day normal galaxies, at epochs that may reach back 95% of the time to the Big Bang.

THE long-standing effort to identify normal galaxies at high redshifts has undergone dramatic progress in recent months. New observations by Steidel *et al.*¹ to magnitude $AB(6930) < 25$ (where $AB(\lambda)$ is the monochromatic magnitude at wavelength λ) have revealed a population of galaxies at redshift $z \approx 3$ and have demonstrated that the Lyman-limit spectral discontinuity and Ly α -forest spectral decrement, which arise owing to photoelectric absorption by neutral hydrogen along the line of sight, together constitute the most prominent spectral signature of very distant galaxies. This result has two important implications. First, it provides a means of identifying high-redshift galaxies. The spectra of high-redshift galaxies are characterized by (1) a complete absence of flux below the Lyman limit and (2) strongly absorbed flux in the Ly α forest. This spectral signature is observable by means of broad-band photometry and must apply irrespective of the underlying spectral properties of the galaxies because it is imprinted by intervening rather than intrinsic material. Second, it allows high-redshift interpretations of low-redshift galaxies to be excluded. The rate of incidence of high-redshift Lyman-limit and Ly α -forest absorbers is sufficiently large that stochastic variations between different lines of sight are essentially negligible. High-redshift galaxies must exhibit the spectral signature of Lyman-limit and Ly α -forest absorption, and any observation to the contrary is sufficient to rule out the possibility of a high redshift.

The Hubble Deep Field (HDF) images, obtained by the Hubble Space Telescope (HST) in December 1995, permit a search for the spectral signature of Lyman-limit and Ly α -forest absorption to magnitudes far fainter than were previously accessible. The images were obtained with the Wide Field and Planetary Camera 2 (WFPC2) through four filters spanning near-ultraviolet to near-infrared wavelengths to a roughly uniform detection limit approaching $AB = 30$ mag. The images are in principle sensitive to galaxies at redshifts as large as $z \approx 7$, beyond which the Lyman limit is redshifted past the response of the WFPC2 filters.

We have estimated redshifts of a large number of galaxies in the HDF images by fitting galaxy spectral-energy distributions, including the effects of intervening Lyman-limit and Ly α -forest absorption, to precise photometry of objects detected in the images. Here we describe the analysis and present a catalogue of 1,683 objects of magnitude $AB(8140) \leq 30$ at estimated redshift ranging from $z = 0$ to $z > 6$. The catalogue is essentially complete to magnitude $AB(8140) < 28$ (1,104 objects), at which 367 objects are at estimated redshift $z = 0-1$, 512 objects are at $z = 1-2$, 135 objects are at $z = 2.0-3$, 54 objects are at $z = 3-4$, 30 objects are at $z = 4-5$, two objects are at $z = 5-6$, and four objects are at $z > 6$. Even if the high-redshift identifications are incorrect, we show by simulations that most real galaxies of these magnitudes and redshifts would be detected and hence that the surface densities we find represent strict upper limits to the actual surface densities. The rapid decline in the number of objects at estimated redshift

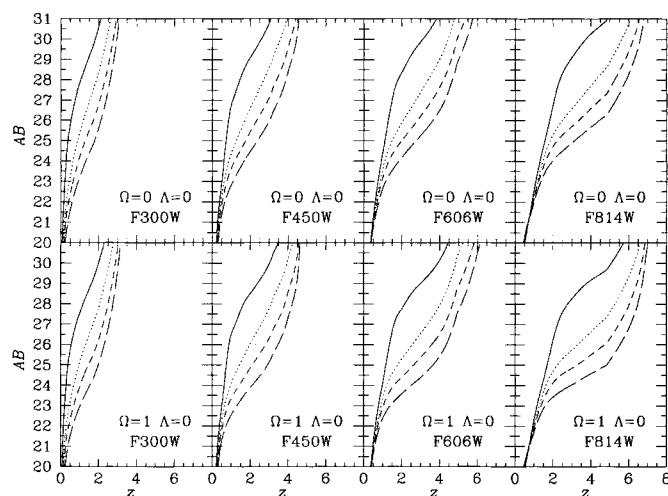


FIG. 1 Expected AB magnitudes of the four galaxy types—E (solid), Sbc (dotted), Scd (short-dashed) and Irr (long-dashed)—through the WFPC2 filters for galaxies of absolute magnitude $M_{AB(4500)} = 20$ ($H_0 = 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$). The top row of panels are for $\Omega = 0$ and the bottom row of panels for $\Omega = 1$. The WFPC2 images are sensitive to galaxies at redshifts as large as $z \approx 7$, beyond which the Lyman limit is redshifted past the response of the F814W filter.

$z > 2$ is therefore significant and tightly constrains models of galaxy formation and evolution (A.Y., K.M.L., A. Campos and A.F.S., manuscript in preparation).

If the estimated redshifts are approximately correct, then the high-redshift objects typically have ultraviolet luminosities $\sim 10^9$ – 10^{10} times the solar luminosity $L_\odot$, sizes ~ 1 kpc, and co-moving spatial densities that vary between 0.05 and 0.01 Mpc^{-3} at redshifts between $z = 2.5$ and $z = 6$. The ultraviolet luminosities and sizes are similar to those of nearby starbursting galaxies, and the co-moving spatial densities are comparable to that of present-day luminous galaxies. (Throughout we adopt a Hubble constant $H_0 = 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and an Einstein–de Sitter cosmology with $\Omega = 1$ and $\Lambda = 0$.) We interpret these objects as galactic or proto-galactic regions of star formation associated with the progenitors of present-day normal galaxies.

Object detection

The first goal of the analysis is to detect objects in the F814W images and to measure their spectral energy distributions in the F300W, F450W, F606W and F814W images. (The spectral sensitivities of these images peak at roughly 3,000 Å, 4,500 Å, 6,060 Å and 8,140 Å, respectively.)

First we obtained and processed the HDF images (produced by the Version 2 drizzle algorithm) made available to the HST archive on 29 February 1996. We considered only the Wide Field Camera images, which are significantly deeper than the Planetary Camera images. We trimmed the images to include only columns and rows 191 to 1970 (because the images are of inferior quality at the edges) and eliminated deviant pixels by setting the value of any pixels that differed by more than 3σ from a local 3×3 pixel median equal to that median (because the images contain a number of single-pixel positive noise spikes or hot pixels). The angular extent of each trimmed image is 71.2×71.2 arcsec², and the total angular area covered by the trimmed images is 4.22 arcmin².

Next, we detected objects in the F814W images using the SExtract program². The object-detection criteria can be set in different ways for different purposes, and we adopted a conservative approach with the aim of achieving high reliability. Specifically, we changed the default parameters of the program by (1) smoothing the images by a gaussian filter of full-width at half-maximum (FWHM) 0.12 arcsec (approximately the width of the point-spread function) to aid the detection of faint sources³, (2) adopting a detection threshold of at least ten contiguous pixels with a signal-to-noise ratio >1.4 each, and (3) eliminating objects that are close to other objects by setting CLEAN_PARAM = 2.0. Criterion (2) is equivalent to a surface brightness limit $\mu_{AB(8140)} < 26.1$ arcsec⁻² over a minimum area of 0.016 arcsec², which corresponds to an isophotal limiting magnitude of $AB(8140) = 30.6$. A total of 1,683 objects were detected, the faintest of which is of total magnitude $AB(8140) = 30.0$.

Next, we tested the completeness of the catalogue by re-applying the detection algorithm for different values of the smoothing and thresholding parameters. The object list does depend on these parameters, but for objects of magnitude $AB(8140) < 28$ we found less than 1% variation for any plausible choices of the parameters. We conclude that the catalogue is essentially complete to magnitude $AB(8140) < 28$.

Next, we determined the local covariance and background surrounding each object in the images. We considered a square region of at least 41×41 pixels surrounding each object (larger regions for larger objects) and excluded pixels associated with any object before measuring the local covariance and background. The local covariances must be used to determine accurate photometric uncertainties because the 'drizzled' HDF images are significantly correlated over adjacent pixels ($\sim 40\%$ with immediate neighbours, $\sim 10\%$ with diagonal neighbours, and less at larger separations).

Last, we measured the spectral-energy distribution of each object detected in the images. We used SExtract segmentation maps to identify the nonoverlapping pixels associated with each object and measured the flux of each object in each of the four images within its unique segmentation aperture. The choice of identical apertures in the four images assures that exactly the same portion of each object is measured in each image, which is essential for establishing meaningful spectral-energy distributions. We subtracted the local background (which was generally negligible) and used the local 3×3 covariance matrices (between each pixel and its immediate and diagonal neighbours) to determine the photometric uncertainties.

Redshift estimation

The second goal of the analysis is to estimate redshifts of all objects by comparing measured and modelled spectral energy distributions.

First, we modelled spectral-energy distributions of galaxies at redshifts $0 < z < 7$. We adopted the galaxy spectra (E/S0, Sbc, Scd and Irr galaxies) of Coleman, Wu and Weedman⁴, extrapolating at wavelengths less than 1,400 Å using the results of Kinney *et al.*⁵ We chose not to apply evolutionary corrections to these spectra because such corrections are uncertain and because the adopted galaxy spectra already span a wide range of spectral properties. We incorporated the effects of intrinsic and intervening Lyman-limit absorption by assuming that galaxies are optically thick to ionizing radiation. This assumption has recently been verified by Leitherer *et al.*⁶ for nearby star-forming galaxies, but in any case the mean free path for intervening Lyman-limit absorption is sufficiently small⁷ to make this assumption valid at all but the lowest redshifts ($z \approx 2.3$) for which the Lyman limit is of interest. We accounted for intervening Ly α and Ly β absorption by applying measurements by Madau⁸ and unpublished measurements by J. Webb of the Ly α -forest flux decrement parameters D_A and D_B . These measurements extend over the redshift range $0 < z < 5$ and were extrapolated to $z = 7$ using a simple fit. We integrated the redshifted spectra with the throughputs of the F814W, F606W, F450W and F300W filters (including system throughputs) to derive the model spectral-energy distributions. Figure 1 shows the expected AB magnitudes (of the four galaxy types) through the WFPC2 filters of a galaxy of absolute magnitude $M_{AB(4500)} = -20$. We also modelled the spectral-energy distribution of an M star using the spectrum of Jacoby, Hunter and Christian⁹, extrapolating at wavelengths greater than 7,500 Å using our own observations.

Next, we constructed redshift likelihood functions of all objects

TABLE 1 Galaxy surface densities

z	Surface density (arcmin ⁻²)			
	$AB(8140) < 25$	$AB(8140) < 26$	$AB(8140) < 27$	$AB(8140) < 28$
0.0–0.5	7.1 (0.6)	13.3 (0.9)	22.0 (1.1)	35.5 (1.4)
0.5–1.0	20.4 (1.1)	28.0 (1.3)	39.1 (1.5)	51.4 (1.7)
1.0–1.5	13.0 (0.9)	23.7 (1.2)	42.7 (1.5)	65.2 (1.9)
1.5–2.0	8.3 (0.7)	18.5 (1.0)	39.6 (1.4)	56.2 (1.8)
2.0–2.5	2.1 (0.3)	8.1 (0.7)	15.2 (0.9)	29.6 (1.2)
2.5–3.0	0.9 (0.2)	1.9 (0.3)	3.1 (0.4)	7.3 (0.6)
3.0–3.5	1.2 (0.3)	2.1 (0.3)	4.5 (0.5)	8.3 (0.7)
3.5–4.0	0.2 (0.2)	1.2 (0.3)	2.6 (0.4)	4.5 (0.5)
4.0–4.5	0.2 (0.2)	0.7 (0.2)	2.8 (0.4)	5.2 (0.5)
4.5–5.0	0.2 (0.2)	0.2 (0.2)	0.5 (0.2)	1.9 (0.3)
5.0–5.5	0.0 (0.2)	0.0 (0.2)	0.2 (0.2)	0.5 (0.2)
5.5–6.0	0.0 (0.2)	0.0 (0.2)	0.0 (0.2)	0.0 (0.2)
>6.0	0.0 (0.2)	0.0 (0.2)	0.2 (0.2)	0.9 (0.2)

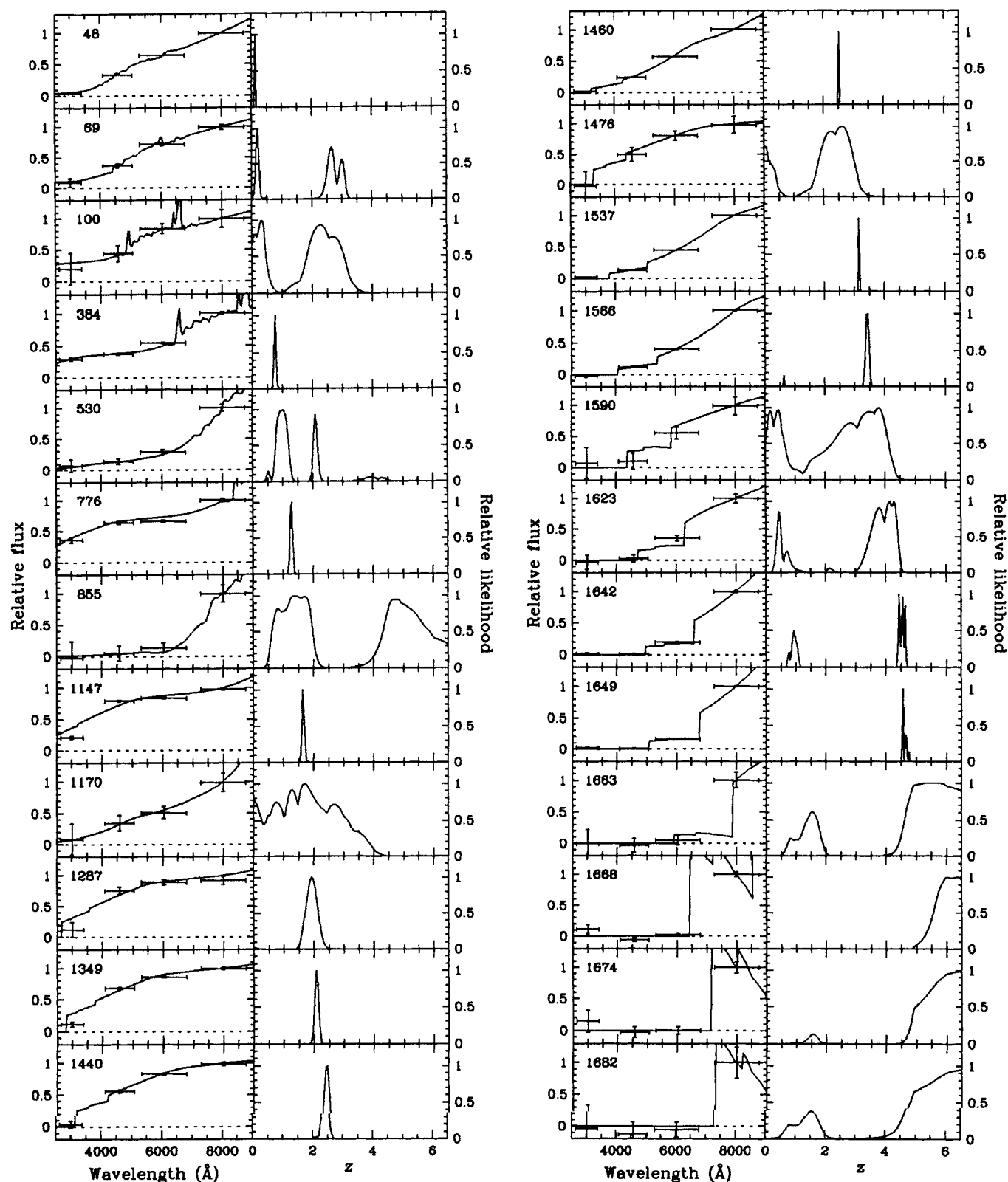


FIG. 2 Sample spectral-energy distributions (left panels of pair) and redshift likelihood functions (right panels of pair) of 24 objects of magnitude $AB(8140) < 28$. For the spectral-energy distributions, vertical error bars show uncertainties, horizontal error bars show bin sizes, and solid curves show best-fit model spectral-energy distributions. Rough uncertainties for the derived redshifts can be estimated by identifying the redshift regions

over which the redshift likelihood functions exceed $\exp(-1/2)$ times the maximum-likelihood values. The redshift likelihood functions are, however, in general quite complicated and often show multiple local maxima. Moreover, they do not take into account the cosmic variance. For these reasons, the uncertainties determined by this method provide only rough indications of the true uncertainties.

in the images by comparing measured and modelled spectral energy distributions. Assuming that flux uncertainties are normally distributed, the likelihood $L(z, T)$ of obtaining measured fluxes f_i with uncertainties σ_i given modelled fluxes $F_i(z, T)$ at an assumed redshift z for spectral type T and normalization A over the four filters $i = 1-4$ is

$$L(z, T) = \prod_i \exp \left\{ -\frac{1}{2} \left[\frac{f_i - AF_i(z, T)}{\sigma_i} \right]^2 \right\} \quad (1)$$

For each object, we maximized equation (1) with respect to spectral type and normalization to determine the redshift likelihood function $L(z)$ and maximized $L(z)$ with respect to redshift to determine the maximum-likelihood redshift estimate. We did not attempt to assign relative abundances to the different spectral types as functions of redshift but simply gave all types equal weight in the redshift likelihood functions.

The result of the analysis is a redshift likelihood function and maximum-likelihood redshift estimate of each of the 1,683 objects detected in the images. Spectral energy distributions and redshift likelihood functions of a representative sample of the objects are shown in Fig. 2 and surface densities of the objects as functions of redshift and limiting magnitude are given in Table 1. The complete catalogue of objects, as well as their spectral-energy distributions and redshift likelihood functions, are available as Supplementary Information, together with optimally added composite spectral energy distributions of the objects. The spectra of the objects fall into distinct classes depending on the estimated redshift. At redshift $z \lesssim 2.3$, the spectra exhibit no significant absorption by intervening material and are similar to the redshifted spectra of present-day galaxies. At redshift $2.5 \lesssim z \lesssim 4$, the spectra are characterized by strong flux in the F814W and F606W images, detectable flux in the F450W images, and no detectable flux in the F300W images. At redshift $4 \lesssim z \lesssim 5.5$, the spectra are characterized by strong flux in the F814W images, detectable flux in the F606W images, and no detectable flux in the F450W and F300W images. At redshift $z \gtrsim 6$, the spectra are characterized by strong flux in the F814W images and no detectable flux in the F606W, F450W and F300W images.

Confirming and corroborating evidence

Several lines of evidence support the redshift estimates obtained by our analysis.

The redshift estimates are in good agreement with the spectroscopic redshifts recently reported by Steidel *et al.*¹⁰ for six high-redshift objects, by Cowie¹¹ for 30 medium-redshift objects, and by Moustakas *et al.*¹² for eight medium- and high-redshift objects, as illustrated in Fig. 3. Of the 44 objects, three high-redshift galaxies have redshifts underestimated by $\Delta z \approx 1$ owing to confusion with ultraviolet emission of other faint galaxies, and four bright ($AB(8140) = 20.5-22.8$) medium-redshift objects have redshifts overestimated by $\Delta z > 1$ because the uncertainties used in equation (1) do not include the cosmic variance. The estimated redshifts of the remaining 37 objects agree with the spectroscopic ones with a root-mean-square (r.m.s.) difference $\Delta z = 0.15$.

The surface density of high-redshift objects found by Steidel *et al.*¹, $0.40 \pm 0.07 \text{ arcmin}^{-2}$ to magnitude $AB(6930) < 25$ at redshift $3 < z < 3.5$, is in good agreement with that found in our analysis, $1.2 \pm 0.5 \text{ arcmin}^{-2}$. Furthermore, the surface densities reported in Table 1 decrease more or less monotonically with increasing redshift, which is in general accord with what is expected for distant galaxies owing to luminosity distance and surface-brightness effects but is not necessarily expected if the redshifts are generally in error.

We conducted two tests to verify that objects detected in the F814W image but not in the other images (and hence identified by the analysis at estimated redshift $z > 6$) are real and not due to noise fluctuations. First, we sought to identify spurious objects in the 'negative' images, which were formed by reversing the sign of each pixel in the images. Using exactly the same procedures that

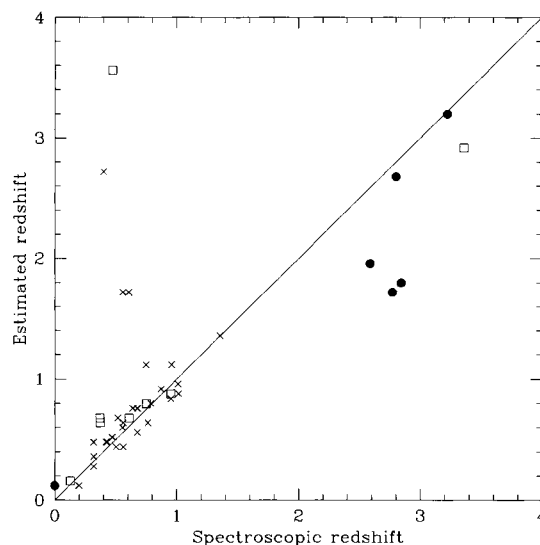


FIG. 3 Redshifts estimated in this Article versus spectroscopic measurements by Steidel *et al.*¹⁰ (filled circles), Cowie (crosses) and Moustakas (open boxes). Of the 44 objects, three high-redshift galaxies have redshifts underestimated by $\Delta z \approx 1$, owing to confusion with ultraviolet emission of other faint galaxies, and four bright ($AB(8140) = 20.5-22.8$) medium-redshift objects have redshifts overestimated by $\Delta z > 1$, because the uncertainties used in equation (1) do not include the cosmic variance. The estimated redshifts of the remaining 37 objects agree with the spectroscopic ones to an r.m.s. difference $\Delta z = 0.15$.

were used on the positive images, we detected only three spurious objects of magnitudes $AB(8140) = 28.5-29.6$ in the negative images, of which only one is at 'estimated redshift' $z > 6$, compared with 16 objects at $z > 6$ in the positive image, of which one, four and 13 are of magnitude $AB(4500) < 27, 28$ and 29 , respectively. This rules out spurious detections due to a symmetric noise distribution. To eliminate the possibility of spurious detections due to a skewed noise distribution, caused by noise added to weak sources below the detection limit, we repeated the analysis with the roles of the F814W and F450W images interchanged. (These two images have approximately the same limiting magnitude; this test cannot be conducted with the F606W image, which is almost a magnitude deeper.) As none of the spectra are expected to peak at $4,500 \text{ \AA}$ and be undetected at higher wavelengths, any detection with 'estimated redshift' $z > 4$ would be spurious. The highest estimated redshift found in the test was, in fact, $z = 3.64$.

The spectra of the extreme objects at estimated redshift $z > 6$ are consistent with redshifted Lyman-limit absorption of high-redshift galaxies but inconsistent with many other interpretations, including lower-redshift galaxies, line-dominated galaxies, and heavily reddened lower-redshift galaxies. The brightest of these objects (object 1,668 in the full catalogue: see Supplementary Information) shows a 3σ lower limit of 12 to the F814W/F606W flux ratio, and the composite spectral energy distribution of these objects shows a 3σ lower limit of 20 to the F814W/F606W flux ratio. No other astrophysical object of which we are aware satisfies these constraints. Specifically, spectrophotometric atlases of galaxies^{13,14} show no galaxy with a spectral decrement anywhere near a factor of 20 over any wavelength range that might be redshifted into the F814W and F606W images, and extreme line-dominated galaxies usually exhibit strong ultraviolet continuum radiation¹⁵.

The possibility that the objects might be heavily reddened low-redshift galaxies is unlikely on several accounts. First, three magnitudes of reddening, which corresponds to about nine magnitudes of extinction in the F814W images, would be required to suppress the F606W images by a factor of 20 relative to the F814W images (assuming a reasonably flat unobscured spectrum). If it were heavily reddened, object 1,668 would have an unobscured

magnitude of $AB(8140) = 26 - 9 = 17$, yet it is smaller than 1 arcsec. Second, if the objects were heavily reddened, then they would be very noticeable at longer wavelengths. Object 1,668 would be of magnitude $AB(19000) \approx 21$ –22. Infrared images of the HDF area of the sky have been checked (L. Cowie, personal communication) and place an approximate, 1σ limit of $AB(19000) \geq 25$ on this object, thus ruling out the possibility of heavy reddening in this case. Third, it is necessary to account for the surface density of the objects, that is, $\sim 1 \text{ arcmin}^{-2}$. Odd, heavily reddened, nearby galaxies cannot serve as prototypes for the galaxies at estimated redshift $z > 6$ unless the density of such local galaxies can be shown to be high enough.

Even if the high-redshift estimates are incorrect, the results of the analysis can be used to set strict upper limits to the surface density of real high-redshift galaxies because such galaxies must exhibit the spectral signature of Lyman-limit and Ly α forest absorption and so must be identified by the analysis as high-redshift objects. Such galaxies would fail to be identified by the analysis as high-redshift galaxies only if they were blocked by bright, foreground galaxies or were incorrectly identified as low-redshift galaxies due to photometric uncertainties. To test both of these effects, we placed 'synthetic' models of all objects of magnitude $AB(8140) < 28$ (matching both the magnitudes and sizes of the real objects) at random locations within the images and sought to recover the redshifts by exactly the same analysis procedures used to identify the real galaxies. Figure 4 shows that the great majority of the objects were recovered at essentially their input redshifts, with an r.m.s. difference $\Delta z = 0.11$, similar to the redshift difference found between the estimated and spectroscopic redshifts. The remaining objects have discordant estimated redshifts owing to photometric uncertainties and confusion with faint sources, but none have estimated redshifts $z > 5$. (Cosmic variance is not an issue as the simulated objects were modelled with the spectral-energy distributions used in the analysis.)

Many of the objects, including some of those with highest estimated redshifts, are clearly spatially resolved, which excludes Galactic stars as possibilities. (Their spectra are, in any case, inconsistent with those of M stars.)

We therefore conclude that the estimated redshifts are probably correct.

Galaxies in the early Universe

If the estimated redshifts are approximately correct, then the high-redshift objects typically have ultraviolet luminosities $\sim 10^9$ – $10^{10} L_{\odot}$, sizes ~ 1 kpc, and co-moving spatial densities that vary between 0.05 and 0.01 Mpc^{-3} at redshifts between $z = 2.5$ and $z = 6$. The luminosities and sizes are modest in comparison with those of luminous galaxies but are similar to those of nearby starbursting galaxies¹⁶. On the other hand, the co-moving spatial

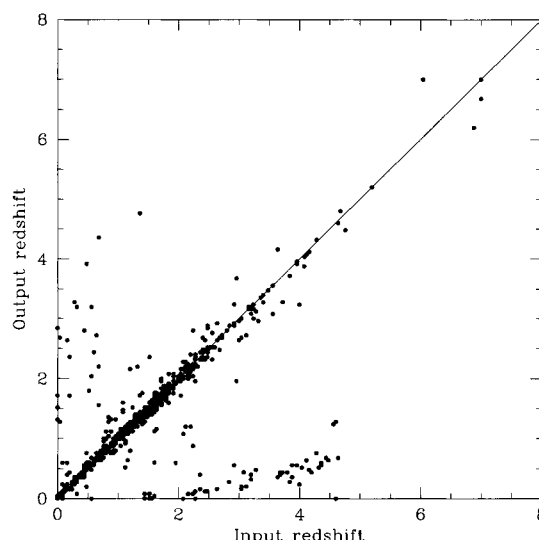


FIG. 4 Output redshifts z_{out} versus input redshifts z_{in} of the synthetic models of the objects. Of the 1,104 objects included into the simulation, 170 (or 15%) were by chance placed on or near other objects and showed a brightening of $\Delta AB(8140) > 0.15$. The 934 remaining objects were recovered with an r.m.s. difference $\Delta AB(8140) = 0.045$ between the input and output magnitudes. Of these, 97 (or 9% of the original sample) were identified by the analysis with redshift differences $z_{\text{in}} - z_{\text{out}} > 0.5$, with typical differences around 2. The remaining 837 (or 76% of the original sample) were identified at output redshifts essentially equal to the input redshifts, with an r.m.s. difference $\Delta z = 0.10$, which is comparable to that between the estimated redshifts and those measured by Steidel *et al.*¹⁰, Cowie¹¹ and Moustakas *et al.*¹².

density of the objects is comparable to or even larger than that of luminous galaxies. The objects therefore appear to represent star formation is small, concentrated, regions rather than in galaxy-sized objects. At the early epochs spanned by the objects, the dynamical timescale of galaxy-sized objects is comparable to the age of the Universe, which suggests that we may be witnessing the first star formation associated with the initial collapse of galaxies. In fact, the rapid decline in surface density as a function of redshift for $z > 2$ tightly constrains models of galaxy formation and evolution (A.Y., K.M.L., A. Campos and A.F.-S., manuscript in preparation). In any event, we interpret the objects as galactic or proto-galactic regions of star formation associated with the progenitors of present-day normal galaxies at epochs reaching back 95% of the time to the Big Bang. $\square$

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The boron isotope ratio in the interstellar medium

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OBSERVATIONS of the abundances of elements provide insight into their production and distribution. The production of light elements (in particular, lithium, beryllium and boron) is dominated by spallation reactions¹, in which cosmic rays break apart more massive nuclei. Models^{2,3} suggest that the $^{11}\text{B}/^{10}\text{B}$ ratio should be about 2.5, but the observed ratio in the Solar System is about 4 (refs 4,5). This has led to the suggestion⁵ that the pre-solar nebula was subjected to bombardment by low-energy Galactic cosmic rays, leading to an overproduction of ^{11}B (ref. 6). Until now, it has not been possible to measure the $^{11}\text{B}/^{10}\text{B}$ ratio in the interstellar medium, because the lines are very weak. Here we present a spectroscopic measurement of the $^{11}\text{B}/^{10}\text{B}$ ratio in the interstellar gas lying between the Earth and the star δ Scorpii, made using the Hubble Space Telescope. Our measured ratio, $3.4^{+1.3}_{-0.6}$, is comparable to the meteoritic value⁵, but somewhat lower. Further measurements will be needed to establish whether the ratio reported here is characteristic of the interstellar medium in general.

Spectra of δ Sco were acquired with the Goddard High-Resolution Spectrograph on the Hubble Space Telescope using the ECH-A grating and the small science aperture providing a nominal resolution of 80,000 or 4 km s^{-1} . The star was selected because a measurement of the interstellar neutral hydrogen column density^{7,8} implied that the resonance line of B II at 1,362 Å would be present at a useful strength. The observing procedure involving eight 80-s sub-exposures at slightly different grating settings in combination with a reduction procedure to minimize noise^{9,10} provided final spectra with continuum signal-to-noise ratios of 350–400. Two settings of the echelle were used: one provides the interstellar B II line at 1,362 Å and the Cu II line at 1,358 Å and the other yields the Ga II line at 1,414 Å.

Although δ Sco was chosen in part because its direction shows rather simple velocity structure for the interstellar gas, it was appreciated that a velocity structure comparable to or even exceeding the predicted isotopic splitting of 3 km s^{-1} (ref. 11) would probably be present; for example, the Na D lines are fully saturated over a width of 5 km s^{-1} (ref. 12). The Cu II and Ga II lines were selected to show the velocity structure in the absence of any appreciable isotope and hyperfine splitting. The gaseous ions B^+ , Cu^+ and Ga^+ should show similar distributions along the line of sight because each is the dominant ion of its element in diffuse clouds and each is incorporated into grains to a similar level¹³. Indeed, inspection of the apparent column-density profiles in Fig. 1c shows close similarities of the Cu II and Ga II profiles, but the B II profile has a red asymmetry not seen in the others, the result of extra absorption centred at $\sim 3\text{ km s}^{-1}$ from the main component. We ascribe this absorption to the less abundant isotope ^{10}B .

The spectra were analysed as follows. The apparent optical depth as a function of velocity¹⁴ was determined. The column density for each pixel (velocity) was obtained directly because the observed lines are quite weak and optically thin. Figure 1c shows the apparent column density profiles for B II, Cu II and Ga II, but scaled to a common maximum column density for easier compar-

ison. A clear excess for B II absorption is apparent at velocities near -7 km s^{-1} ; we ascribe this excess absorption to the isotope ^{10}B . For a more detailed analysis the component structure in the Cu II and Ga II lines was obtained, yielding absorption strength, position and width¹⁵; the Cu II and Ga II absorption profiles required three independent interstellar components. The derived velocity separations, velocity widths and relative absorption strengths were adopted in a synthesis of the B II feature using a code that minimized r.m.s. deviations. Both boron isotopes were needed to get an acceptable fit. Thus, in addition to the parametrization of global absolute velocity and total column density, the isotope ratio and the isotope shift were determined through a synthesis with six components. The results are displayed in Fig. 1a, b. The combined analysis based on weighted averages of the two syntheses indicates an isotope ratio of $3.4^{+1.3}_{-0.6}$ (1σ) and an isotope shift of $(13.7 \pm 3.5) \times 10^{-3}\text{ Å}$. The isotope shift for the line at wavelength 1,362 Å is consistent with predictions of $13.3 \times 10^{-3}\text{ Å}$ from large-scale theoretical computations with an inherent precision of 1% (ref. 11). Our astronomical measurements for the isotope shift provide the first empirical confirmation of the theoretical prediction. Our analysis, which included the predicted hyperfine splitting of $0.45 \times 10^{-3}\text{ Å}$, was not sensitive to this small effect.

The interstellar boron abundance is also of interest for studies of chemical evolution in our Galaxy^{16–18}. Our measurements indicate a B II column density of $(8.5 \pm 0.7) \times 10^{10}\text{ cm}^{-2}$ for the gas toward δ Sco. Through the use of previous H I (ref. 7) and H₂ (ref. 8) observations of this star, a boron abundance of $(7.9 \pm 1.7) \times 10^{-11}$ is obtained, where the uncertainty includes the accuracy of the hydrogen observations. This abundance is consistent with all other interstellar determinations^{17,18} when the same sources for H I and H₂ are used: λ Ori ($(9.2 \pm 4.1) \times 10^{-11}$), ι Ori ($\leq 1.2 \times 10^{-10}$), κ Ori ($(9.3 \pm 4.9) \times 10^{-11}$) and ζ Oph ($(8.9 \pm 2.8) \times 10^{-11}$). The interstellar boron abundance is considerably lower than the abundances found in disk stars^{19,20} (2×10^{-10}), in the solar photosphere^{21,22} ($(4 \pm 2) \times 10^{-10}$) and in meteorites^{4,23} ($(7.6 \pm 0.7) \times 10^{-10}$). The origins for the order-of-magnitude differences in the boron abundance are not known at present.

The interstellar isotope ratio of $3.4^{+1.3}_{-0.6}$ that we have found for boron is formally equal to the standard terrestrial ratio, the NBS standard 951 (ref. 5) of 4.04558. Bulk meteoritic material also exhibits the terrestrial value. This concordance suggests that one should seek a general explanation of the ratio, which has not changed appreciably from the interstellar ratio prevailing at the time that the solar system formed, 4.5 Gyr ago. The recent discovery of γ -ray lines from excited C and O nuclei toward Orion²⁴ requires a 30-fold increase (over extrapolated values) in the flux of low-energy ($E \leq 30\text{ MeV}$) Galactic-cosmic-ray C and O nuclei in this interstellar environment. The enhanced flux of low-energy C and O would, if present in most or all star-forming regions, provide boron by spallation reactions with an isotope ratio higher than the 2.5 provided from relativistic Galactic cosmic rays (GCRs). This solution to the B isotope problem is now favoured by many^{25–28}. This and other invocations of Orion-like low-energy cosmic rays are capable of fitting the boron isotope ratio of 4. (In the relativistic GCRs, boron has an isotope ratio²⁹ of 3.5 ± 0.2 . This observed ratio is not necessarily in conflict with the predicted ratio of 2.4 from spallation driven by the GCRs. The latter refers to GCR protons colliding with interstellar C, N and O nuclei to produce low-velocity B nuclei. The observed B nuclei in the GCRs result from GCR C, N and O nuclei hitting interstellar protons but, as these high-energy B nuclei are thermalized, they are largely destroyed and the isotope ratio is altered.)

Ion-probe measurements of chondrules in four chondritic meteorites of differing histories show a variation of the isotope ratio from 3.84 to 4.25 that is correlated with the Be and B concentrations⁵. The variation is attributed by the discoverers to

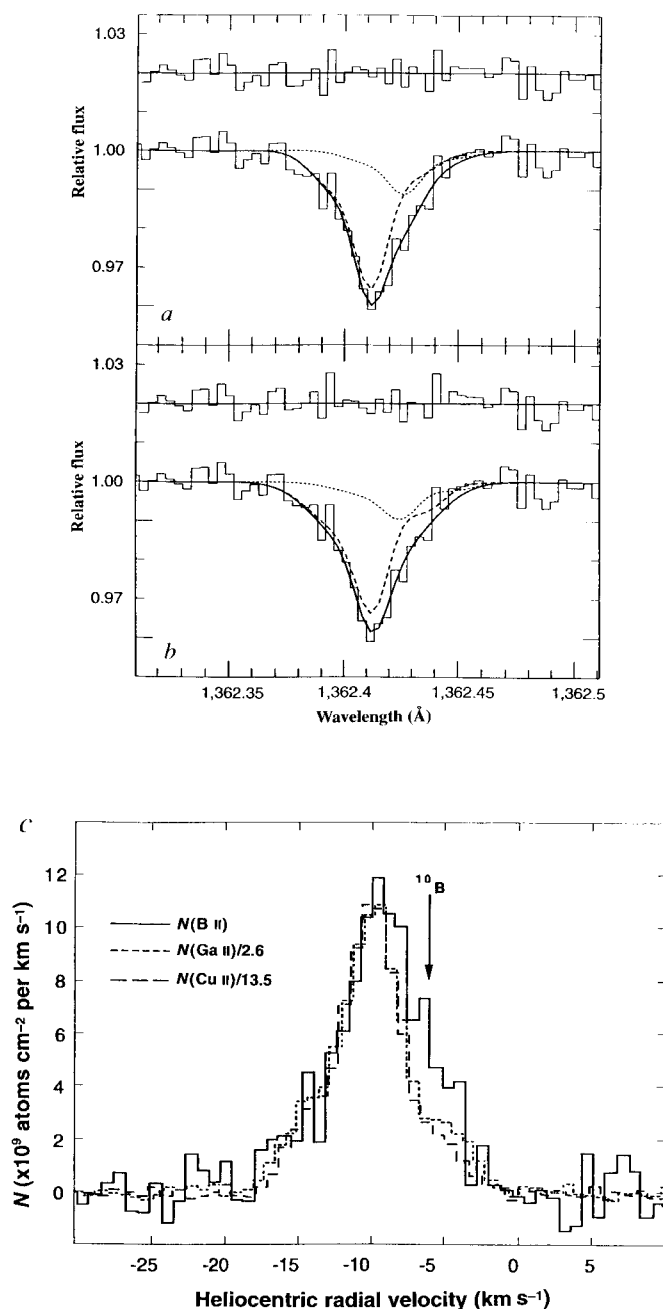


FIG. 1. *a, b* Absorption from interstellar B toward δ Sco. The observations are indicated by the histograms and the profile syntheses by a solid line; the upper panel illustrates the synthesis from fits to the Cu II line, and the lower panel the Ga II line. The contributions from ^{11}B (broken lines) and ^{10}B (dotted lines) are shown. The qualities of the fits appear as traces of the difference between the observed and synthesized profiles displayed beneath it. The fits involved a Voigt absorption profile based on oscillator strengths given by Morton³², convolved with a gaussian instrumental profile of width $17 \times 10^{-3} \text{ \AA}$. From the Cu II fit, a total boron column density of $(8.3 \pm 0.7) \times 10^{10} \text{ cm}^{-2}$ and a $^{11}\text{B}/^{10}\text{B}$ ratio of $3.2^{+1.8}_{-0.8}$ were obtained. The Ga II profile yielded respective values of $(8.7 \pm 0.7) \times 10^{10} \text{ cm}^{-2}$ and $3.6^{+2.9}_{-0.9}$. The syntheses yielded isotope shifts of $(14.5 \pm 4.5) \times 10^{-3} \text{ \AA}$ ($(12.5 \pm 5.5) \times 10^{-3} \text{ \AA}$) for the Cu II (Ga II) fits. In the fits to the B II feature, the error bars were computed from the uncertainty in equivalent width ($W_\lambda = (1.36 \pm 0.11) \times 10^{-3} \text{ \AA}$). The post-fit r.m.s. deviation was 0.95 of the r.m.s. deviation for the continuum outside the fitted region, thereby indicating a reduced χ^2 of 0.9. *c*, The apparent column-density profiles¹⁴ for B II (solid lines), Ga II and Cu II, normalized to the profile for B II. The profiles for Ga II and Cu II should be multiplied by 2.6 and 13.5 respectively. The excess column density associated with ^{10}B is indicated.

chemical heterogeneities in interstellar gas (and dust) that were transmitted to the meteoritic chondrules by accretion of interstellar grains with different isotope ratios. Exposures to Orion-like cosmic rays to differing levels were invoked to raise the isotope ratio to differing levels above the value of 2.4 predicted from the relativistic GCRs. Quite possibly the different exposures occurred in the cloud from which the Sun and other stars later formed. Alternatively, several gas clouds exposed to different levels were later mixed. In either case one might expect differences in the isotope ratio within a diffuse cloud and between clouds. Our result encompasses the 10% spread in the meteoritic isotope ratios. Detection of such differences between diffuse clouds is a major challenge for the future. Formally our present result, which extends considerably beyond the spread in the meteoritic values, allows an isotope ratio as low as 2.8 or as high as 4.7. The lower limit is effectively consistent with relativistic GCRs as the sole contributor of boron isotopes. The upper limit possibly calls for a contribution of ^{11}B from neutrino-induced spallation within type II supernovae.

Because several sites of uncertain efficacy are responsible for the nucleosynthesis of the five stable nuclides comprising Li, Be and B, we have at present too few data to claim a full understanding of the origins of these elements. For instance, other routes considering selective destruction of light nuclides (say ^{10}B over ^{11}B) have been suggested³⁰ to account for the boron isotope ratio. Our measurement of the interstellar B isotope ratio helps but additional measurements of this ratio are needed. We expect to report on other lines of sight shortly, thereby providing information on the intrinsic variation in the interstellar ratio. Of especial interest would be measurements in more distant clouds, including the gas in the Magellanic Clouds, so that the theoretical models can be applied to environments with a range of CNO abundances and star formation rates. Detection of interstellar Be and additional results on the interstellar B abundance would help constrain models of light element nucleosynthesis because these light elements have different sources. There is the interesting but remote prospect of measuring the isotope ratio in stars of different ages, metallicities and locations by using the B I 2,090 Å line for which the isotope shift is $25 \times 10^{-3} \text{ \AA}$ (ref. 31). The B II 1,362 Å line in stellar spectra is a less attractive candidate because the isotope shift is smaller and the lines in hot stars are generally much broader than they are in the cool stars in which the B I line is observed. □

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Suppression of discrete aurorae by sunlight

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IT HAS long been assumed that aurorae, like stars, are present during daylight, but hidden by the high surface brightness of the sky. The brightest aurorae are discrete curtains of light created by beams of electrons accelerated in the Earth's magnetosphere. Many theories¹ have been proposed to explain discrete aurorae, but the one that provides the best match to recent observations² invokes a positive feedback mechanism between the conductivity of the ionosphere, which is enhanced by the injection of electrons, and the current carried by these same electrons^{3–5}. Here we present a statistical study of electron precipitation events by using nine years of charged-particle data from weather satellites. We find that the beams of accelerated electrons that cause the discrete aurorae occur mainly in darkness: the winter hemisphere is favoured over the summer hemisphere, and night is favoured over day. We also find that discrete aurorae rarely occur in the presence of diffuse aurorae, which provide sufficient conductivity to support the electric currents between the magnetosphere and the ionosphere. Taken together, these observations strongly support the view that ionospheric conductivity is the key factor controlling the occurrence of discrete aurorae.

Charged particles from space, mainly electrons and protons, constantly precipitate along geomagnetic field lines into the Earth's upper atmosphere at high latitudes. Most of this precipitation, and the auroral airglow it produces, is diffuse, representing a direct dumping of the various space plasma populations that surround the Earth. But at times the electrons show clear signs of magnetic field-aligned acceleration, by hundreds of eV or occasionally even a few tens of keV. The result is a characteristic profile in the electron spectra⁶, sometimes loosely called 'monoenergetic', because of the sharp energy-flux peak at the acceleration energy. Such accelerated beams of electrons are now known⁶ to correspond to the brighter and more intense type of aurorae, namely discrete aurorae, which are bright arcs, rays or curtains, narrow in latitude and elongated in longitude. Discrete aurorae can be studied optically only in dark conditions, whereas accelerated electrons can be measured by satellite at any time.

In seeking a test for the ionospheric conductivity feedback hypothesis, we realized that if it was correct the mechanism should operate much better in darkness than in sunlight, where conductivity is already high. We were further motivated to investigate the role of sunlight by noting that the magnetosphere–ionosphere system is dynamic at all local times, yet intense aurorae are concentrated in the dusk–midnight sector. Here we report results from a massive statistical study of accelerated-electron precipitation events with data from the US Air Force Defense Meteorological Satellite Program (DMSP) series satellites. Details about the DMSP particle detectors and a description of the algorithm to identify monoenergetic electron acceleration events² can be found elsewhere. In all, nine years of data encom-

passing five satellites and more than 152 million distinct spectra were analysed.

The role of sunlight was investigated by calculating the probability of observing electron acceleration events as a function of solar zenith angle (0° means the Sun is overhead, while at 90° the Sun is at the horizon). The probability is defined as the number of spectra taken in a bin that showed signs of electron acceleration divided by the number of spectra taken in that bin, thus giving a 'snapshot' probability for a precise site and a single second of time. The probability that an observer standing on the ground within the auroral zone and watching the skies (in darkness) will see discrete aurorae is higher than suggested at first glance by the figures that follow. This is because such an observer can monitor a large region of the sky simultaneously, and can do so for longer than one second.

The results for all discrete aurorae above $5.0 \text{ erg cm}^{-2} \text{ s}^{-1}$ (the threshold of visibility with the unaided eye is $\sim 1 \text{ erg cm}^{-2} \text{ s}^{-1}$) are

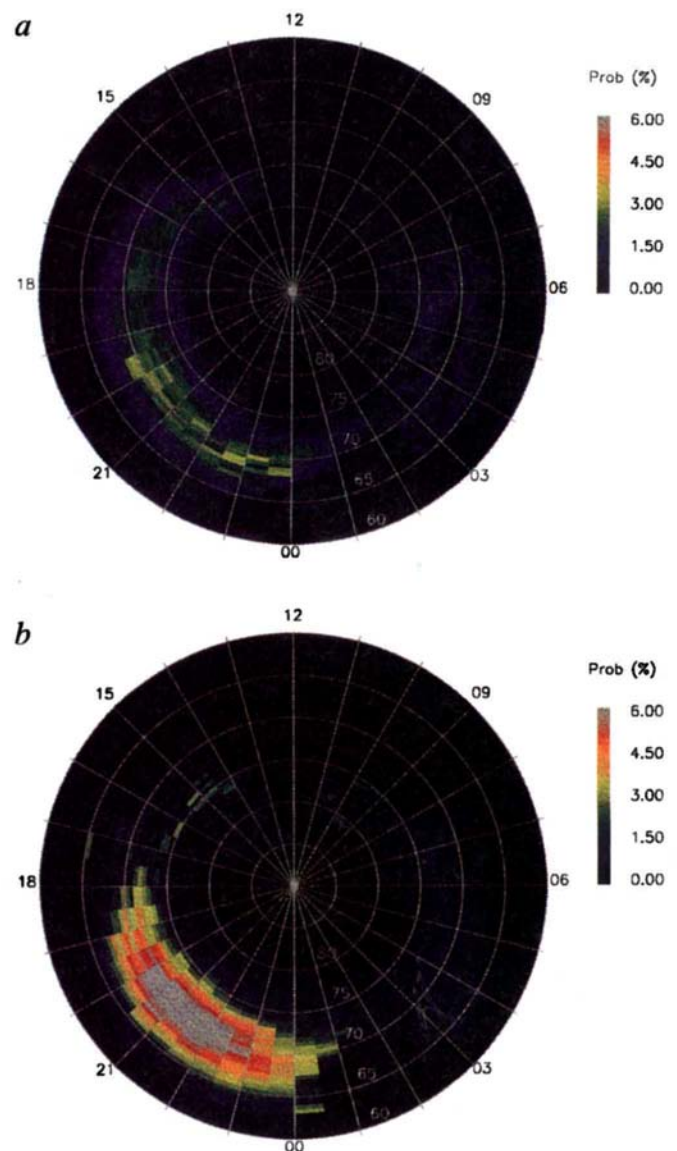


FIG. 1 The probability, as a function of magnetic latitude (from 60° to 90°) and local time, of observing the accelerated electrons that cause discrete aurorae, for observations made from December 1983 to November 1992 inclusive. All events above $5.0 \text{ erg cm}^{-2} \text{ s}^{-1}$ (a level easily visible to the unaided eye) are included. *a*, Under sunlight conditions (solar zenith angle < 85°); *b*, in darkness (solar zenith angle > 110°).

shown in Fig. 1a, b, corresponding to conditions of zenith angle $>110^\circ$ and $<85^\circ$ respectively. A surprisingly large difference is observed, with discrete aurorae suppressed under sunlit conditions. The difference is especially pronounced in the dusk-midnight sector, where intense discrete aurorae are found to be most common; here the ratio of discrete aurorae under sunlit versus dark conditions is 3.

Because the solar zenith angle at a given local time depends on season and geographical latitude, one would expect to get the same effect by selecting days near the summer and winter solstices, and indeed this occurred in our data set (intense discrete aurorae occur dominantly in the winter bin). But because the effect is about as strong for the full set of data as for those days around solstice we believe it is not a strictly seasonal phenomenon. For either method of event selection, the probability of observing an intense discrete aurora at any one latitudinal cut is about three times that in the favoured conditions (sunlit or summer) than in unfavourable conditions (darkness or winter).

As mentioned above, we wished to investigate the effect of sunlight on aurorae to test the ionospheric conductivity feedback mechanism^{3,4}. In this theory, a region of enhanced precipitation becomes a region of enhanced conductivity (because the precipitation ionizes the upper atmosphere), which causes more current to flow between the magnetosphere and ionosphere, further enhancing the precipitation. Such conditions can lead to the type of field-aligned electron acceleration that causes discrete aurorae and is studied here⁵. As originally proposed and considered so far, the role of pre-existing ionospheric conductivity from other sources was not considered. Although an intense arc can indeed generate as much or more conductance than even full sunlight, the ionosphere is primed for the feedback instability only when the background conductivity is low. Under such circumstances any precipitation immediately represents the dominant source of conductance.

To quantify and make explicit the relationship between conductivity and discrete arcs, we calculated the ionospheric conductivity from its main sources, namely sunlight and diffuse aurorae. For sunlight we used an empirical equation relating solar zenith angle to Pedersen conductivity developed by Rasmussen *et al.*⁷. For calculating the contribution of diffuse electron precipitation we used our own data base of nine years of DMSP

satellite observations, with the events of intense field-aligned acceleration removed, along with the equations given by Robinson *et al.*⁸. The result, calculated under equinox conditions (00 UT on 21 March), is shown in Fig. 2. Superimposed on the conductivity measurements is the well-known statistical location of field-aligned currents out of the ionosphere⁹. Such currents must be complete circuits; they therefore require closure and thus a conducting channel within the ionosphere. Field-aligned currents driven by the magnetosphere out the ionosphere can be supported by ionospheric conductivity from one source or the other except in the dusk-midnight sector. After midnight the conductivity is supported by hot electrons from the Earth's plasma sheet, whose curvature and gradient drift towards dawn places them precisely into the region of upward currents. For this reason, even under winter conditions, the post-midnight sector has fewer aurorae than pre-midnight.

Discrete auroral arcs are impressive displays that have been studied optically for centuries by means up to and including satellite images, although always in darkness. Discrete aurorae can now be understood to be the means whereby the magnetosphere-ionosphere system establishes the conductivity needed to support currents between the magnetosphere and ionosphere. Hot electrons from the nightside plasma sheet combined with sunlight supply conductivity in all local time sectors except from dusk to midnight. Because currents are also required in this region, the mechanism of auroral arcs creates keV-energy electrons from the relatively cold (a few hundred eV) population available, and thereby the necessary conductivity. Indeed, out of 22 mechanisms proposed for creating discrete aurorae¹, only the ionospheric conductivity feedback mechanism explains our result that discrete aurorae are suppressed under sunlit conditions. □

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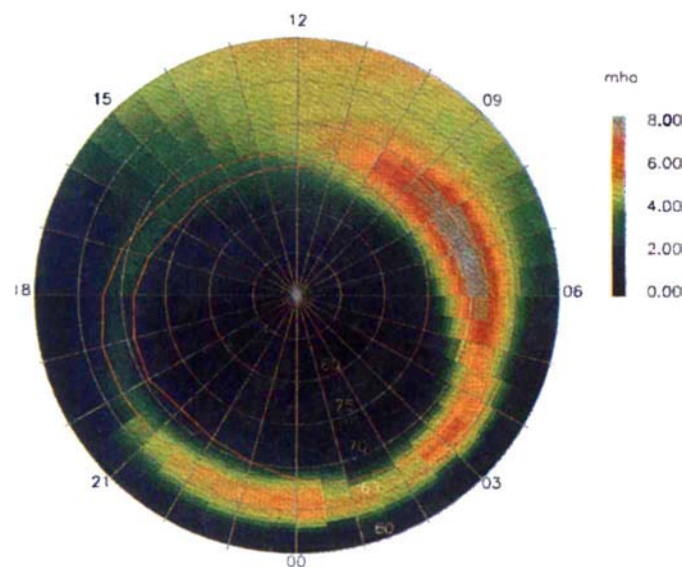


FIG. 2 The height-integrated Pedersen conductivity including contributions from diffuse (unaccelerated) aurorae and sunlight at equinox. The red lines give the average position of field-aligned currents out of the ionosphere. Discrete aurorae occur mainly where upward currents are required and conductivity is low.

Turbulent cascades in foreign exchange markets

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THE availability of high-frequency data for financial markets has made it possible to study market dynamics on timescales of less than a day¹. For foreign exchange (FX) rates Müller *et al.*² have shown that there is a net flow of information from long to short timescales: the behaviour of long-term traders (who watch the markets only from time to time) influences the behaviour of short-term traders (who watch the markets continuously). Motivated by this hierarchical feature, we have studied FX market

dynamics in more detail, and report here an analogy between these dynamics and hydrodynamic turbulence³⁻⁸. Specifically, the relationship between the probability density of FX price changes (Δx) and the time delay (Δt) (Fig. 1a) is much the same as the relationship between the probability density of the velocity differences (Δv) of two points in a turbulent flow and their spatial separation Δr (Fig. 1b). Guided by this similarity we claim that there is an information cascade in FX market dynamics that corresponds to the energy cascade in hydrodynamic turbulence. On the basis of this analogy we can now rationalize the statistics of FX price differences at different time delays, which is important for, for example, option pricing. The analogy also provides a conceptual framework for understanding the short-term dynamics of speculative markets.

A flow of energy from large to small scales is one of the main characteristics of fully developed homogeneous isotropic turbulence in three spatial dimensions²⁴. It provides a mechanism for dissipating large amounts of energy in a viscous fluid. Energy is pumped into the system at large scales of the order of, say, metres (by a moving car or a flying aeroplane) or kilometres (by meteorological events), transferred to smaller scales through a hierarchy of eddies of decreasing sizes, and dissipated at the smallest scale—of the order of millimetres in the above examples. This cascade of kinetic energy extending over several orders of magnitude generates a scaling behaviour of the eddies and manifests itself in a scaling of the moments $\langle (\Delta v)^n \rangle$ of Δv as $(\Delta r)^{\zeta_n}$ (refs 5,6). Here the angle brackets $\langle \rangle$ denote the mean value of the enclosed quantity and Δv is the difference of the velocity component in the direction of the spatial separation of length Δr . Under the assumption that the eddies of each size are space-filling and that the downward energy flow is homogeneous, $\zeta_n = n/3$ (ref. 8). The probability densities $P_{\Delta v}(\Delta v)$ are then scale invariant. This means that if the velocity differences are normalized by their respective standard deviation, the resulting standardized probability densities do not depend on Δr . But for $n > 3$, experimentally determined values of ζ_n follow a concave curve definitely below the $(\zeta_n = n/3)$ -line (Fig. 2b). The dependence of the standardized probability density on Δr also provides evidence that eddies of a given size are not space-filling but rather fluctuating in space and time in a typical intermittent way (Fig. 1b).

Our analyses of FX markets are based on a data set provided by Olsen and Associates containing all worldwide 1,472,241 bid-ask quotes for US dollar-German mark exchange rates which have emanated from the interbank Reuters network from 1 October 1992 until 30 September 1993. From these data we have determined the probability densities of price changes $P_{\Delta x}(\Delta x)$ with time delays Δt varying from five minutes up to approximately two days, which are displayed in Fig. 1a. In comparison, Fig. 1b shows the analogous turbulent probability densities $P_{\Delta v}(\Delta v)$, which exhibit the same characteristic features. Using the probability density $P_{\Delta x}(\Delta x)$, the information acquired by observing the market after a time Δt can be quantified as $I(\Delta t) = -\int P_{\Delta x}(\Delta x) \log P_{\Delta x}(\Delta x) d(\Delta x)$. It turns out that the dependence of this information on Δt is directly related to the scaling of the variance of Δx with Δt . In turbulence, on the other hand,

the variance of the velocity differences at a distance Δr is proportional to the mean energy which is contained in an eddy of size Δr . This further supports the proposed analogy between energy and information.

Given the analogy between turbulence and FX market dynamics, we expect the moments of FX price changes to scale with the time delay as $\langle (\Delta x)^n \rangle \propto (\Delta t)^{\zeta_n}$. Scaling has already been reported for the mean absolute values of FX returns in ref. 9 and by Evertsz in ref. 1, and for the second moments of the variations of the Standard & Poor's 500 economic index in ref. 10. For FX

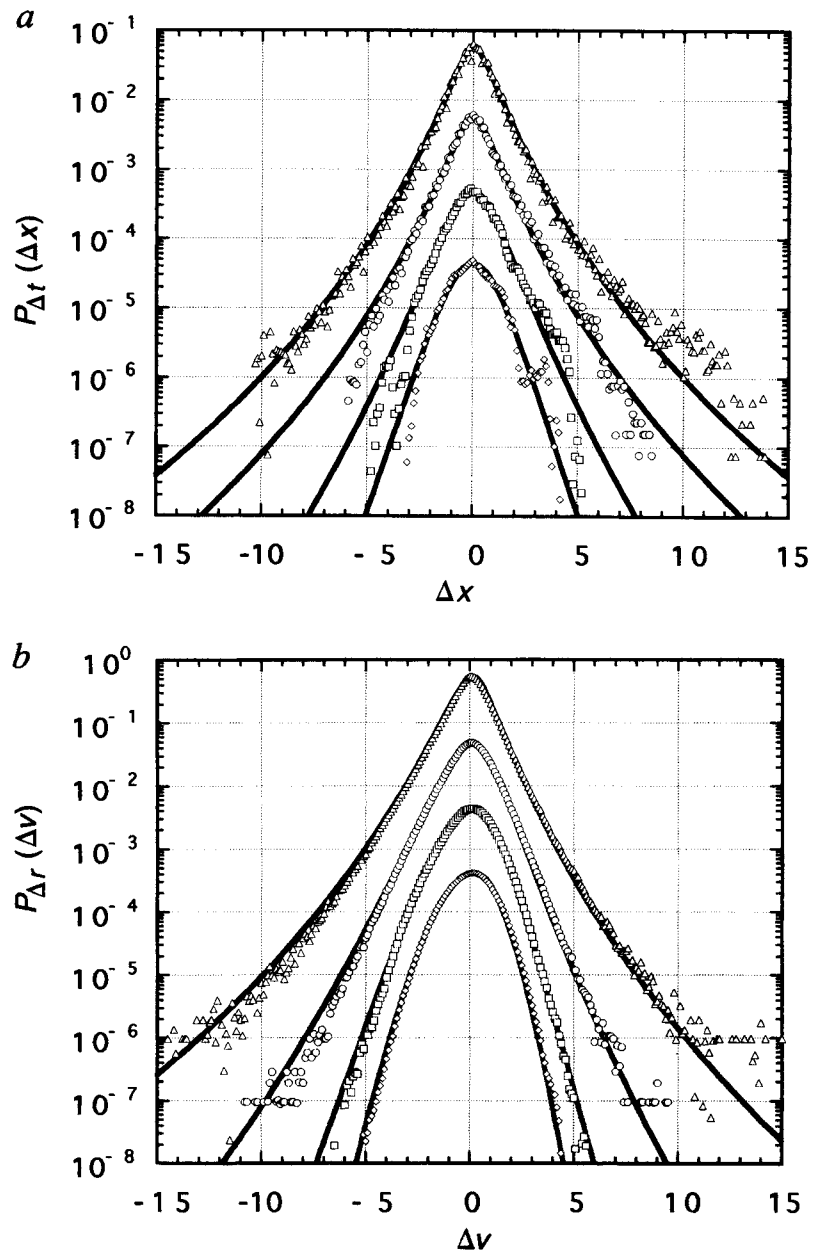


FIG. 1 a, Data points: standardized probability density $P_{\Delta x}(\Delta x)$ of price changes $\Delta x = x(t) - x(t + \Delta t)$ for time delays $\Delta t = 640$ s, 5,120 s, 40,960 s, 163,840 s (from top to bottom). The middle prices $x(t) = (x_{\text{bid}}(t) + x_{\text{ask}}(t))/2$ have been used (data provided by Olsen & Associates (see text)). The probability density has been calculated in a similar way as ref. 10. Full lines: results of (least-squares) fits carried out according to ref. 7; $\lambda^2 = 0.25, 0.23, 0.13, 0.06$ (from top to bottom). For better visibility, the curves have been vertically shifted with respect to each other. Note the systematic change in shape of the densities. b, Data points: standardized probability density $P_{\Delta v}(\Delta v)$ for a turbulent flow with $\Delta r = 3.3\eta, 18.5\eta, 138\eta, 325\eta$ (data taken from ref. 13, $R_i = 598$. Here η is the Kolmogorov scale, where viscous dissipation occurs. Full lines: results of (least-squares) fits carried out according to ref. 7; $\lambda^2 = 0.19, 0.10, 0.04, 0.01$.

market data, it has also been observed that the kurtosis, which is the ratio of the fourth moment and the squared variance, decreases with increasing time delay, that is, the tails of the probability density lose weight^{9,11}. Figure 2a shows the higher-order moments of the FX price changes. They scale for time delays Δt varying from about five minutes up to several hours. As in turbulence, the scaling exponents ξ_n depend on the order of the moments in a nonlinear way. Moreover, the ξ_n of the FX data are close to the ξ_n of turbulent data (Fig. 2b). This is also in agreement with the observation that the shapes of the turbulent and FX probability densities, $P_{\Delta v}(\Delta v)$ and $P_{\Delta x}(\Delta x)$, depend on their respective scale parameters in a similar way: both show a decrease of kurtosis with increasing scale parameter¹² (Fig. 1a and 1b).

Even though the probability densities $P_{\Delta x}(\Delta x)$ and $P_{\Delta v}(\Delta v)$ are in principle determined by their moments, this is of little help in practice because errors drastically increase when calculating higher-order moments from observed data. In turbulence, for different experimental situations, the change in shape of $P_{\Delta v}(\Delta v)$ has been successfully parametrized using a method motivated by the energy cascade^{7,13,14}. The standardized probability density is approximated by a superposition of gaussian densities with log-normally distributed variances. The variance λ^2 of this log-normal distribution is a measurable form parameter containing information on the energy cascade¹⁵. It is the only parameter that must be

TABLE 1 Correspondence between fully developed three-dimensional turbulence and FX markets

Hydrodynamic turbulence	FX markets
Energy	Information
Spatial distance	Time delay
Laminar periods interrupted by turbulent bursts (intermittency)	Clusters of low and high volatility
Energy cascade in space hierarchy $\langle (\Delta v)^n \rangle \propto (\Delta r)^{\xi_n}$	Information cascade in time hierarchy $\langle (\Delta x)^n \rangle \propto (\Delta t)^{\xi_n}$

adjusted to the data. Applying this method to the FX market data yields a surprisingly good fit (Fig. 1a). Although the data in the centre dominate the fit, the agreement is reasonable also as regards the tails of the probability densities as long as the time delay is shorter than about two days. This is in contrast to fits with Levy distributions which significantly deviate in the tails¹⁰. The parameter λ^2 , which measures the spread of the variances of the superimposed gaussians (that is, the variance of the log-normal distribution), decreases linearly with $\log \Delta t$ (Fig. 3). This result further confirms the similarity of the statistical behaviour of FX markets with the classical picture of turbulence as given by Kolmogorov⁵⁻⁷. In particular, it verifies the scaling of the moments, $\langle (\Delta x)^n \rangle \propto (\Delta t)^{\xi_n}$, in an independent way.

An important aspect of turbulent flows is their intermittent behaviour, that is, the typical occurrence of laminar periods which are interrupted by turbulent bursts. In FX markets this corresponds to clusters of high and low volatility¹², which give rise to relatively high values of the probability densities $P_{\Delta x}(\Delta x)$ both in the centre and the tails. This particular aspect is well reproduced for time delays smaller than two days by the proposed log-normal superpositions of gaussians. However, long interruptions of the trading process (particularly during weekends) affect the probability densities and lead to deviations from the expected shape. Hence, we conclude that the range which is governed by the information cascade has an upper limit. We note that in turbulence also there is an upper length scale, where the energy cascade sets in and beyond which scaling fails.

Different types of models have been proposed to describe the statistical characteristics of price differences of financial indices as, for example, the behaviour of the volatility. Prominent approaches are models using mixtures of distributions¹⁶⁻¹⁸ and ARCH/GARCH-type models^{19,20} (see, for example, the overview in ref. 21). But these studies do not address the scaling behaviour of the probability distributions of FX price changes.

It is unlikely that there is a set of a few partial differential equations (like the Navier Stokes equations in hydrodynamics) which might serve as a model of FX market dynamics. More striking is the similarity of the two phenomena, which is accounted for by the existence of a cascade in both cases. This has prompted us to introduce concepts of turbulence in the description of FX

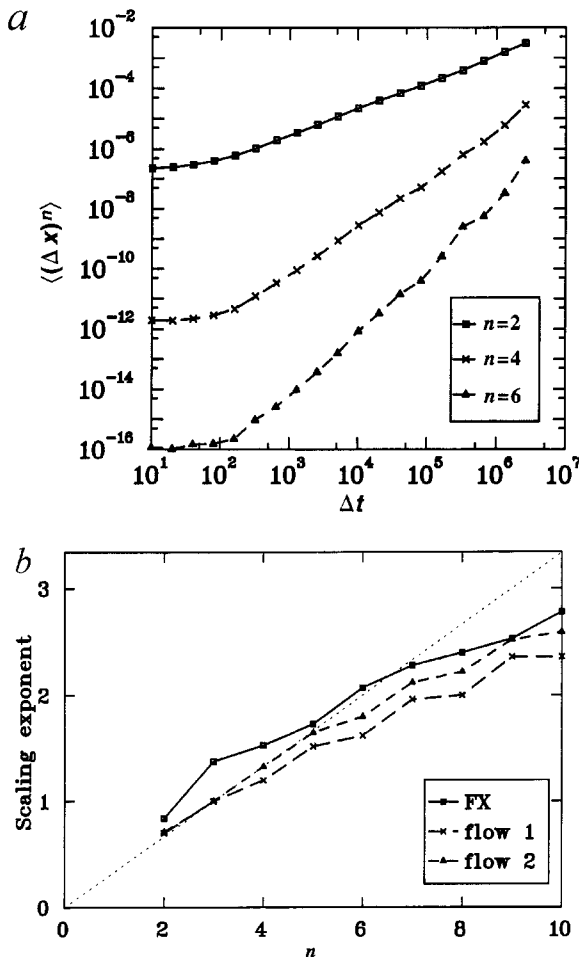


FIG. 2 a, Scaling of moments $\langle (\Delta x)^n \rangle$ with $n = 2, 4$ and 6 . b, n -dependence of the scaling factors ξ_n and ζ_n for the n th moments of the probability densities of FX price changes (squares) and hydrodynamic velocity differences taken from ref. 22 (crosses) and ref. 23 (triangles). Note the same qualitative deviation of all curves from a straight line.

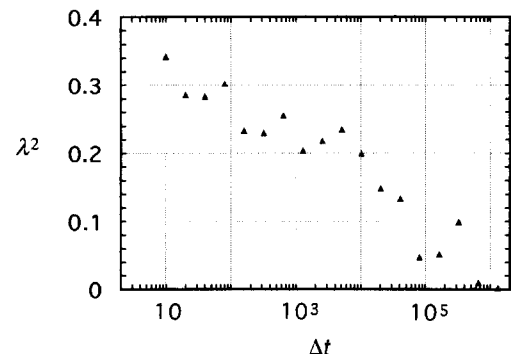


FIG. 3 Dependence of the form parameter λ^2 on Δt (in seconds).

markets, in particular that of an information cascade. Table 1 summarizes the corresponding notions. How far this analogy goes still has to be shown. In any case, we have reason to believe that the qualitative picture of turbulence that has developed during the past 70 years will help our understanding of the apparently remote field of financial markets. □

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Identification of a new type of electronic state in the magnetoresistive orthomanganites

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PEROVSKITES of composition $\text{Ln}_{1-x}\text{A}_x\text{MnO}_3$ (where Ln and A are rare-earth and alkaline-earth elements respectively) have become the focus of scientific and technological interest because of their extraordinary electronic and magnetic properties. For example, compositions with $x = 0.3$ exhibit an unusual combination of ferromagnetism and good electrical conductivity¹; moreover, the observation² of a 'colossal' magnetoresistive response near the Curie temperature (T_C), which can be changed dramatically by altering the strength of the Mn–O–Mn interactions^{3,15}, points to potential practical applications for these materials. Recent resistivity studies⁴ of these materials have suggested that the accepted 'tight-binding' band description⁵ of the conduction electrons below T_C may be inadequate and that strong interactions between the electrons and dynamic local lattice distortions associated with the Mn sites may be important. Here we report resistivity and thermopower measurements at high pressure, which indicate a more exotic origin for the

electronic properties of these materials. We find that below T_C the conduction electrons lose their localized character and condense into extended electronic states that exhibit no energy dispersion. We attribute the formation of this new state to the strong coupling of the conduction electrons to cooperative oxygen vibrations along the Mn–O bond axes.

Hwang *et al.*³ increased the strength of the Mn–O–Mn interactions of compounds with $x = 0.3$ (that is, a fixed $\text{Mn}^{4+}/\text{Mn}^{3+}$ ratio) by decreasing the bond-length mismatch as given by the tolerance factor $t = (\text{R–O})/\sqrt{2}(\text{Mn–O})$, where R–O (R = $\text{Ln}_{0.7}\text{A}_{0.3}$) and Mn–O are the mean equilibrium bond lengths as calculated from room-temperature ionic radii. In the range $0.96 \leq t \leq 0.97$, Hwang *et al.*³ found a first-order transition from polaronic conduction (mobile electronic species 'dressed' in a local lattice deformation) above T_C to a strongly reduced resistivity in the canted-spin ferromagnetic phase below T_C . The canted-spin ferromagnetism can be understood as a competition between antiferromagnetic superexchange interactions via virtual charge transfer between half-filled π -bonding t_2 orbitals on opposite sites of an oxygen atom, that is, $\text{Mn}t_2\text{–O–Mn}t_2$ interactions varying as $\cos \theta_{ij}$, and ferromagnetic double-exchange interactions via a rapid (with respect to spin-relaxation time) real charge transfer of σ -bonding e electrons from a high-spin Mn^{3+} to a Mn^{4+} ion, which varies as $\cos(\theta_{ij}/2)$ (ref. 5); θ_{ij} is the angle between spins on neighbouring Mn atoms.

We report an investigation of the compound $(\text{La}_{0.25}\text{Nd}_{0.75})_{0.7}\text{Ca}_{0.3}\text{MnO}_3$ that was chosen to have $t \approx 0.96$; at this value of t the colossal magnetoresistance is at a maximum, and the temperature dependence of the resistivity below T_C changes from semiconductive to metallic with increasing t . We have used hydrostatic pressure to increase, at fixed chemical composition, the strength of the Mn–O–Mn interactions from that for $t \approx 0.96$ at ambient pressure.

Figure 1A shows the resistivity ρ versus temperature T , $\rho(T)$, data for several hydrostatic pressures P . We highlight four features: (1) The maximum in the $\rho(T)$ curve occurs at the Curie temperature T_C (ref. 3); the data show a pressure dependence $dT_C/dP > 0$. (2) A strong thermal hysteresis indicates a first-order transition occurs at T_C as previously noted³; we plot in Fig. 1B, *a* thermal hysteresis and T_C for different pressures. (3) Whereas $\rho(T)$ below T_C is commonly reported to be metallic, our compound cannot be a conventional metal at these temperatures for two reasons. First, the dramatic drop in the resistivity on lowering T below T_C does not obey a power law and, second, the magnitude of $\rho(T)$ below T_C , $\rho(30\text{ K})$ for example, decreases by more than three orders of magnitude as the pressure is increased to 15 kbar (Fig. 1B, *b*). In contrast, the polaronic conductivity at temperatures $T > T_C$ changes by only a factor of two in the same pressure range. Moreover, at lowest temperatures the resistivity increases with decreasing temperature (Fig. 1B, *c*). (4) The high-pressure limit of the low-temperature resistivity $\rho(0)$ is of the same order as the high-temperature limit of $\rho(T)$.

The conductivity $\sigma = ne\mu$, where n is the density of carriers of charge e with mobility μ , is classified into two regimes: (1) carriers that move diffusively with a mobility $\mu = eD/kT$, where the diffusion coefficient $D = D_0 \exp(-\Delta G_m/kT)$ contains a motional enthalpy ΔH_m in the free energy ΔG_m and (2) itinerant (conduction-band) carriers with an effective mass m^* and a mobility $\mu = e\tau_c/m^*$ that tunnel without activation energy until they are scattered after a mean free time τ_c by a lattice aperiodicity, scattering by acoustic phonons giving $1/\tau_c \propto T$. Neither process has a mobility that is strongly pressure-dependent as can be seen, for example, for the polaronic conduction above T_C in Fig. 1B, *a*. Therefore, the extremely strong pressure dependence of $\rho(T)$ below T_C is indicative of a sensitive increase in the density of mobile charge carriers with increasing pressure below T_C .

In support of this deduction, we have demonstrated elsewhere⁶ that mobile charge carriers are progressively trapped as T is lowered to T_C . In Fig. 1A we plot a Voigt function matched to a $\rho(T)$ curve below T_C . The Voigt function is normally used to

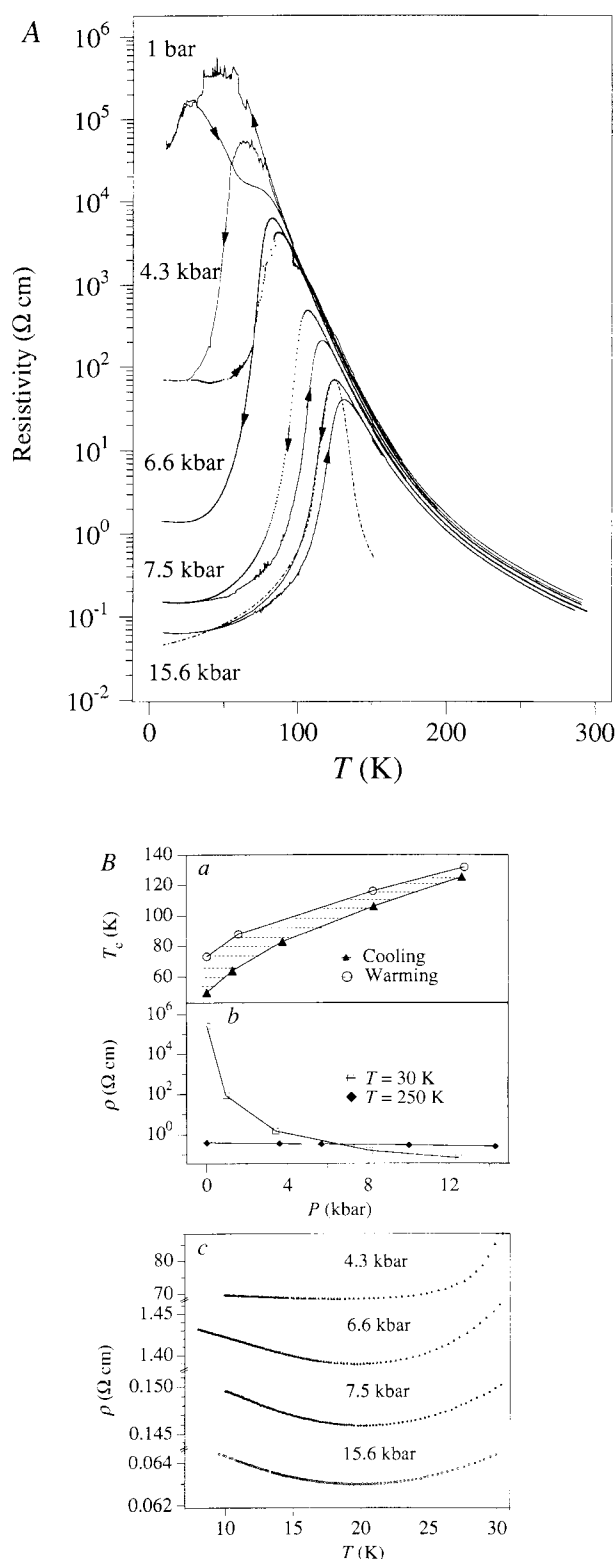


FIG. 1 A, Resistivity versus temperature for several pressures. The sample preparation and the self-clamped pressure cell for measurement have been described elsewhere⁶. A four-probe Montgomery configuration¹⁴ was used to measure the resistivity of our polycrystalline sample. The original ambient-pressure results obtained before measurement under pressure were recovered on release of the pressure. Dot-dashed curve is a Voigt-function fit to the $\rho(T)$ of $P = 15.6$ kbar below T_C . B, a, Thermal hysteresis and T_C as a function of pressure. The pressures used in the figure were measured at T_C . B, b, Resistivity versus pressure at 30 K and 250 K. B, c Expanded data for $T \leq 30$ K on a linear scale. (Note breaks in y-axis scale)

describe variation in intensity of a spectroscopic peak, and its good fit to $\rho(T)$ below T_C is consistent with the density of mobile charge carriers increasing continuously with decreasing temperature over a wide temperature interval. The first-order phase change at T_C thus marks a change from a phase where charge carriers are progressively trapped out to one where they are progressively released with decreasing temperature; it is distinguishable from a classical metal-insulator transition.

The thermopower α provides a probe of the character of the electronic states above and below T_C . Figure 2 shows $\alpha(T)$ curves for the sample under several hydrostatic pressures. At ambient pressure, the resistance was too high, Fig. 1A, to measure $\alpha(T)$ at temperatures < 110 K. However, pressure reduces the resistance at T_C , and $\alpha(T)$ becomes measurable where ρ drops below $10^3 \Omega$ cm. We call attention to three features. First, a maximum in $\alpha(T)$ at a temperature ($T_{\max}$) above T_C decreases as T_C increases with increasing pressure. Second, a thermal hysteresis at T_C is observed. Third, below T_C , $\alpha(T)$ drops to a value near zero that is independent of both temperature and pressure.

Above T_C , $\alpha(T)$ is well described by a polaronic phase in which holes occupy distinguishable sites⁶. The abrupt drop in $\alpha(T)$ on lowering the temperature through T_C indicates that the charge carriers condense from a random distribution of small polarons into a state that eliminates the polaronic distinguishability between Mn^{3+} and Mn^{4+} ions. A value near zero for α that is independent of temperature and pressure is incompatible with any type of polaron formation. On the other hand, a conventional metallic state exhibits an $\alpha(T) \propto kT/\epsilon_F$, where ϵ_F is the Fermi energy with respect to the top of the conduction band. According to the resistivity below T_C , ϵ_F should be very small, which would give a large slope in an α versus T plot. Figure 2, however, shows a temperature-independent $\alpha(T) \approx 0$ below T_C . From the general expression for the thermopower⁷, a value of ≈ 0 for α means that if the conduction electrons have a well defined wavevector $\mathbf{k}$, there is no asymmetry of the one-electron energy dispersion $\epsilon(\mathbf{k})$ in an energy interval kT on either side of ϵ_F . However, the lack of a pressure dependence of the thermopower below T_C (despite a resistivity change with pressure of more than three orders of magnitude) shows that the unusual character of the extended states remains independent of the density n of mobile holes. We are forced to the conclusion that the holes are condensed into a degenerate state having no energy dispersion. The mobility of the mobile holes below T_C remains undefined. However, the thermopower indicates that it is not thermally activated. From Fig. 1A, the lowest resistivity obtained in our high-pressure limit in these experiments is still far from that expected for metallic conduction.

Independent evidence for a correlation by breathing-mode oxygen vibrations of occupied and empty states associated with Mn ions on opposite sides of an oxygen atom was pointed out for the single-valent system $\text{LaMn}_{1-x}\text{Ga}_x\text{O}_3$ where, at higher values of x , a cooperative, static Jahn-Teller deformation is suppressed⁸; ferromagnetic $\text{Mn}^{3+}-\text{O}-\text{Mn}^{3+}$ superexchange interactions require a dynamic correlation of half-filled and empty e orbitals associated with the Mn^{3+} ions. The gradual increase in $\rho(T)$ with decreasing T below 20 K (Fig. 1B, c) may reflect lowering phonon numbers or a softening of the optical mode that couples to the electrons.

The lack of an $\epsilon(\mathbf{k})$ dispersion indicates the presence of a ground-state degeneracy which is normally lifted in solids by a static Jahn-Teller deformation, polaron formation, stabilization of a charge-density wave, or superconductivity. However, a strong, dynamic coupling of electrons to optical-mode vibrations to give vibronic states locates the conducting particles in a joint electron-phonon space. Vibronic coupling lifts the degeneracy of the electronic states while leaving degenerate the quasi-particles (charge carriers) of the entire system⁹.

Zener's original formulation of the double-exchange mechanism¹⁰ correctly envisaged a collapse of the polaronic motional enthalpy ΔH_m at T_C , but his prediction of a $\rho(T) \propto T/T_C$ below T_C is incompatible with our data. Moreover, the extended wave

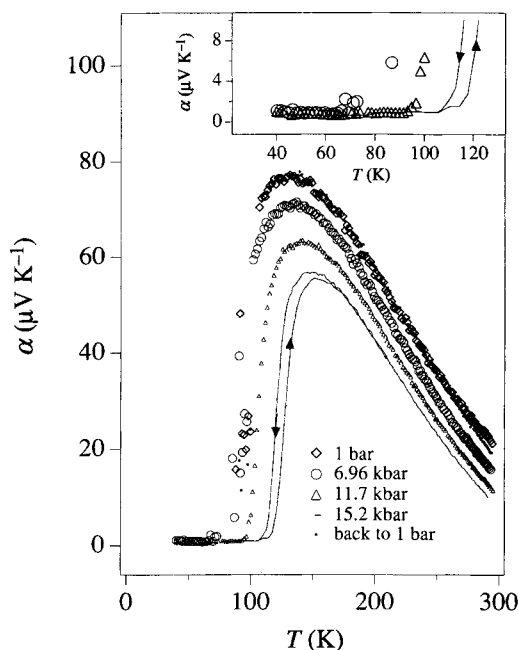


FIG. 2 Thermopower versus temperature for several pressures. Inset, expanded data for $T < T_C$. The contribution to the thermopower from the copper leads was subtracted from the $\alpha(T)$ data. This contribution was obtained by measuring $\alpha(T)$ for a copper oxide superconductor below its critical temperature $T_C \approx 120$ K; it was shown to be pressure-independent, and the temperature dependence above 120 K was taken from the literature. In our apparatus, the systematic error with respect to the true value of α is $\pm 0.4 \mu\text{V K}^{-1}$, but the apparatus does not give a correct measurement of $\alpha(T)$ for $T < 30$ K.

packet proposed by Coey *et al.*¹¹, which is analogous to a large magnetic polaron, is also incompatible; it would give a relative large α below T_C .

On the other hand, models based on tight-binding band theory also fail. The tight-binding band model used by de Gennes⁵ has a bandwidth proportional to the spin-dependent resonance integral

$$t_{ij} \approx \varepsilon_\sigma \lambda_\sigma^2 \cos \phi \cos(\theta_{ij}/2) \quad (1)$$

where ε_σ is a one-electron energy, λ_σ is the covalent-mixing parameter between the e orbitals associated with the Mn ions and the σ -bonding oxygen p orbitals $O:p_\sigma$, and $(180^\circ - \phi)$ is the Mn–O–Mn bond angle. In this formulation, the bandwidth increases with three parameters. First, decreasing ϕ , that is, increasing tolerance factor t ; second, pressure, which increases λ_σ , and third, long-range magnetic order through $\cos(\theta_{ij}/2)$. The temperature T_{max} of maximum thermopower marks the temperature of maximum hole trapping; $T_{\text{max}} > T_C$ signals holes beginning to be released from the traps with the onset of short-range magnetic order. With a tight-binding band model, trapping can occur in Anderson localized states at the top of the band, such localization resulting from dopants that perturb the periodic potential by energies comparable to a bandwidth. Broadening of the bands by magnetic order would lower the Fermi energy relative to the mobility edge so as to release trapped holes¹². However, such a model has previously been associated only with band theory and predicts an $\varepsilon(k)$ dispersion as ε_F crosses the mobility edge. In our system, an increasing bandwidth appears to transfer Anderson localized states to a degenerate set of vibronic states.

The peak in the colossal magnetoresistance versus temperature relation³ is due to a shift of T_C by the magnetic field. In our example, where the tolerance factor is chosen to be $t \approx t_c$ (where t_c is a critical value below which $\rho(T)$ shows no drop at T_C), a large magnetoresistance also occurs below T_C where $\rho(T)$ shows a large pressure dependence. Release of trapped holes is responsible for

the magnetoresistance as well as the dramatic pressure effects; this release is clearly enhanced by increasing t_{ij} in equation (1). For the orthomanganites with $t \gg t_c$, t_{ij} is increased by reducing ϕ in equation (1), and metallic behaviour has been observed below T_C . In this case, the resistivity approaches a normal dependence on pressure and magnetic field¹³. Neither a tight-binding band description nor a polaronic model can describe the unusual pressure and temperature dependence of $\rho(T)$ and the collapse of thermopower that we observed for a composition with $t \approx t_c$; the data thus identify a novel electronic state stabilized below T_C . We suggest that a vibronic coupling occurs between the electron and phonon systems that is analogous to what might be expected with a dynamic, cooperative Jahn–Teller mode. □

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Magnetic thin films of cobalt nanocrystals encapsulated in graphite-like carbon

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A VARIETY of crystalline materials have now been encapsulated in nanometre-scale graphitic cages^{1–8} and tubules^{9–11}. For magnetic materials, encapsulation should serve the dual role of protecting air-sensitive particles against degradation and reducing the magnetic coupling between individual particles, thus potentially opening the way for applications of these composite materials as ultra-high-density magnetic recording media^{12,13}. But to realize this potential, the materials must be available in the form of thin, smooth films, with precise control of the size of the individual magnetic crystallites. Here we report the fabrication and characterization of magnetic thin films, consisting of cobalt nanocrystals encapsulated in graphite-like carbon cages, that meet these structural requirements.

We have taken an approach to encapsulating cobalt nanocrystals in graphite-like carbon that is different from the commonly used arc-discharge method. We co-deposit cobalt and carbon by using ion-beam sputtering with subsequent annealing. A Kaufman-type ion gun¹⁴ is used to produce an argon ion beam with an energy of 1.5 keV and a diameter of ~ 30 mm. A 60×60 mm cobalt target (99.9%) is placed adjacent to a 60×60 mm carbon

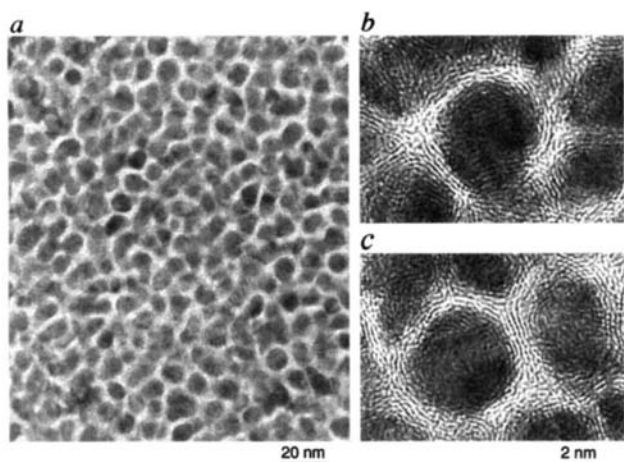


FIG. 1 a, TEM image of Co-C film co-sputtered at 200 °C, then annealed at 250 °C for one hour and subsequently at 300 °C for one hour. b, c, Magnified images.

target (99.9%). By moving the ion beam around the boundary between the two targets, we can control the Co-C composition.

In our experiment, the deposition chamber was pumped by a turbo-molecular pump to a base pressure of $\sim 1 \times 10^{-9}$ torr. The Ar gas pressure during deposition was 2×10^{-4} torr. Cleaved NaCl substrates were mainly used for transmission electron microscopy (TEM) observations. For magnetic measurements and atomic force microscopy (AFM) observations, the Co-C films were deposited through a 5-mm-diameter mask onto glass substrates (Corning 0211). For both types of substrates, 5-nm-thick pure carbon film was first deposited by using a carbon target on the opposite side of the target holder; Co and C were then co-deposited by immediately flipping the holder to the Co-C two-target side. The substrate temperature was varied from room temperature to 300 °C. The growth rate was ~ 3 nm min $^{-1}$. After deposition, the films were lamp annealed in a vacuum ($< 1 \times 10^{-5}$ torr). The composition of the films was evaluated by Auger electron spectroscopy (AES); the film morphology and crystal structures were examined by TEM and electron diffraction; and the surface roughness was measured by AFM. The in-plane magnetic properties were measured at room temperature by using a vibration sample magnetometer.

The grain size and the crystal structure of the as-deposited Co-C films depended on the substrate temperature and the carbon concentration in the film. We found that the high-carbon-concentration films (40–60 at.%) deposited at 200 °C were

amorphous but still had grain-like structures with grain sizes of 3–10 nm. We also found that the initial grain sizes did not change during the succeeding annealing and that the amorphous grains transformed into crystalline cobalt encapsulated in graphite-like carbon. Figure 1 shows TEM images of Co-C film co-sputtered at 200 °C, then annealed at 250 °C for one hour and subsequently at 300 °C for one hour. The film thickness as measured by a DEKTAK surface profiler was 38 nm. The average concentration of carbon in the film as measured by AES was 46 at.%. No impurity elements were detected by AES depth profiling analysis. Nanoparticles were uniformly dispersed in the film, as shown in Fig. 1a; the average diameter of the particles was estimated to be 7.2 nm with a fairly sharp size distribution (with a standard deviation of 1.7 nm). As shown in Fig. 1b and c, lattice images with about 0.2-nm spacing are clearly seen inside the nanoparticles, indicating that these nanoparticles are single-crystalline. The cobalt nanocrystals are completely isolated from each other by graphite-like carbon; lattice images of the carbon with about 0.36-nm spacing are also clearly seen. The thickness of the graphite-like carbon was ~ 2 nm. Yogi and Nguyen reported that physical gaps of 2–5 nm appeared to be sufficient to decouple the magnetic grains¹³. Murakami *et al.* reported that grain separation of 1–2 nm by SiO₂ layers remarkably weakened the exchange coupling¹⁵. Therefore we can expect that the 2-nm thick graphite-like carbon layers can also significantly reduce the exchange coupling between cobalt nanocrystals.

The electron diffraction profile obtained from the film in Fig. 1 is plotted against lattice spacing d and $1/d$ in Fig. 2. All the major observed peaks can be assigned to those of cobalt with a hexagonal close-packed (h.c.p.) structure, whose peak positions in the X-ray powder data are indicated by filled arrows with their plane indices. A small amount of face-centred cubic (f.c.c.) cobalt may exist because the position of the shoulder at ~ 0.18 nm coincides with that of the cubic (200) peak. The broad peak at around 0.36 nm corresponds to the graphite (002), although the peak position is shifted a little from that of bulk graphite (0.34 nm), indicated by the open arrow. Although the weak peak at ~ 0.24 nm may imply that a slight amount of cobalt carbide exists in the film, no distinct peaks corresponding to the known cobalt carbide phases can be seen in Fig. 2. This is reasonable because cobalt carbide is known to be unstable at temperatures higher than about 300–500 °C, transforming into stable cobalt and graphite phases^{16–18}. From these results, we conclude that Co-C co-deposited film annealed at 300 °C mainly consists of h.c.p. cobalt nanocrystals wrapped by several layers of graphite-like carbon.

Konno and Sinclair studied the crystallization of co-sputtered Co-C amorphous films by annealing in a wide Co-C composition range¹⁸. They found fine (5–10 nm) crystalline Co grains at high carbon concentrations (≥ 40 at. %), but the carbon matrix was amorphous phase and the Co grains had no ferromagnetic nature. Tajima and Hirano studied the structure and magnetic properties of Co-C co-sputtered thin films, but did not report any Co

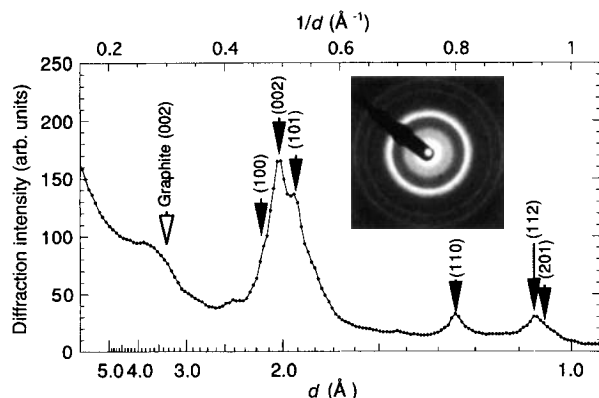


FIG. 2 Electron diffraction profile obtained from the film in Fig. 1 plotted against lattice spacing d and $1/d$. Inset, the electron diffraction pattern.

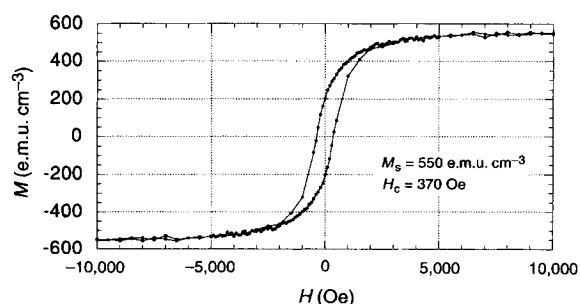


FIG. 3 In-plane M - H curve of a 38-nm-thick Co-C thin film co-deposited on a Corning 0211 glass substrate under the same sputtering conditions as those for the film in Fig. 1, then annealed at 350 °C for one hour in a vacuum.

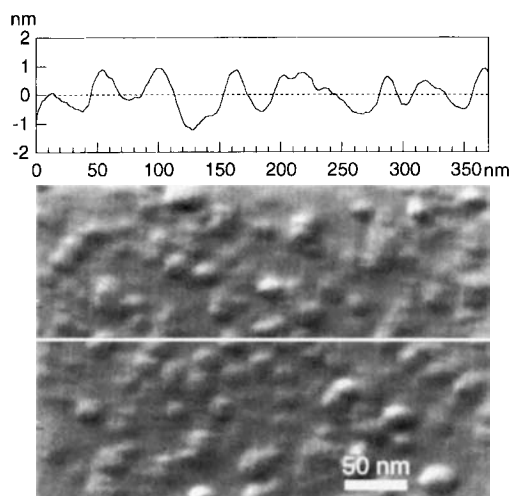


FIG. 4 Bottom, AFM image of sample used for magnetic measurement. Scale bar, 50 nm. The cross-sectional profile (top) corresponds to the white line in the AFM image.

nanocrystals or graphite-like carbon formation¹⁹. Therefore, to achieve the formation of ferromagnetic Co nanocrystals isolated and embedded in a graphite-like carbon matrix, an optimum combination of Co–C composition, substrate temperature, and annealing procedure is essential.

Although carbon-based magnetic thin films have been studied for both magnetic recording media¹⁹ and recording heads^{20,21}, little is known about their possible use as the former. To investigate the potential of sub-10-nm Co nanocrystals isolated by graphite-like carbon for ultra-high-density recording media, we examined their magnetic properties and surface smoothness. A 38-nm-thick film was co-deposited on a Corning 0211 glass substrate under the same sputtering conditions as those for the film in Fig. 1. This film was annealed first at 250 °C for one hour, then at 300 °C for one hour and finally at 350 °C for one hour in a vacuum. After thinning from the backside of the glass substrate, direct TEM observation showed that the film morphology after the whole annealing process was the same as that in Fig. 1, with an average cobalt nanocrystal size of 8 nm. At each annealing, we measured the in-plane M – H curve at room temperature and found that saturation magnetization (M_s) and coercivity (H_c) increased as the annealing temperature increased. The in-plane M – H curve obtained after annealing at 350 °C for one hour is shown in Fig. 3. M_s and H_c were 550 e.m.u. cm⁻³ and 370 Oe, respectively, from Fig. 3. The obtained M_s of 550 e.m.u. cm⁻³ is at the same level as those of cobalt-based alloy media, such as CoPtCr/Cr (300–500 e.m.u. cm⁻³)²², which is one of the candidates for ultra-high-density recording¹³. Considering the volume fraction of Co nanocrystals in the film, the M_s of individual Co nanocrystals is close to that of bulk Co (1,400 e.m.u. cm⁻³). It has been reported that a high H_c of 2,500 Oe is required for future ultra-high-density recording media¹². While the obtained H_c of 370 Oe is too small, there is a good possibility of improving it by optimizing the fabrication processes because H_c is sensitive to many structural parameters, such as the orientation, shape, internal stress, and defects of the Co nanocrystals.

Figure 4 is an AFM image of the sample used for magnetic measurement. The mean deviation from the average height was estimated to be 0.45 nm. The surface roughness of this film is also similar to that of one of the best media surfaces (0.5–0.6 nm; ref. 23), which is good enough for practical use. A thin film of Co nanocrystals isolated and embedded in graphite-like carbon thus has great potential for ultra-high-density magnetic recording media. □

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Reduced meltwater outflow from the Laurentide ice margin during the Younger Dryas

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THE cause of the Younger Dryas cold event, which interrupted the last deglaciation, is still a matter of debate¹. A prevalent hypothesis, proposed by Broecker *et al.*² is that the abrupt climate change was driven by a decrease in the rate of North Atlantic Deep Water production, triggered by a sudden dilution of North Atlantic surface water in response to the diversion of Laurentide ice-sheet melt water from the Mississippi drainage system to that of the St Lawrence river. Here we investigate the feasibility of this triggering mechanism by reconstructing sea-surface temperature, salinity and sea-ice cover records for the outlet of the Gulf of St Lawrence into the North Atlantic Ocean. These reconstructions—based on dinoflagellate-cyst assemblages^{3,4} in sediment cores from the region—show reduced meltwater runoff, low temperatures and extensive sea-ice cover during the Younger Dryas, dated here between 10,800 and 10,300 BP. Meltwater pulses did occur before and after the Younger Dryas event: as early as 11,700 BP, during the development of the Champlain Sea in the St Lawrence Lowland, and afterwards, until ~10,100 BP. At the resolution of our salinity proxy (0.7‰), the meltwater pulses preceding the Younger Dryas did not affect sea-surface salinity off the shelf break. These constraint on the meltwater outflow through the St Lawrence drainage system do not support the triggering mechanism of the Broecker *et al.* hypothesis², unless the North Atlantic thermohaline circulation is more sensitive to small salinity changes than most models suggest²³.

The Laurentide ice retreat during the last deglaciation was accompanied by the discharge of large amounts of melt water into the North Atlantic and adjacent basins⁵. The Mississippi river constituted a major outlet for this melt water until the eastward axis of the Great Lake–St Lawrence river axis was free of ice^{6,7}. Stable isotope data provide evidence for a dilution of surface water in the Gulf of Mexico related to meltwater discharge

through the Mississippi until about 11,000 BP (ref. 2). There is also field evidence for ice fluctuations along the southern Laurentide ice margin, for changes in Lake Agassiz water levels, and for a southward to eastward shift of its overflow by about 11,000 BP (refs 8,9). On these grounds, it has been proposed that the routing of southern Laurentide ice melt water through the St Lawrence outlet at ~11,000 BP was responsible for lower sea-surface salinities in the northern North Atlantic, and thus for the turnoff of the North Atlantic-driven thermohaline circulation and the consequent global cooling of the Younger Dryas event^{2,10}. Although not unreservedly accepted¹, this hypothesis is still acknowledged by default as the actual impact of meltwater routing through the St Lawrence outlet on sea-surface conditions in the North Atlantic is not constrained. We therefore decided to investigate the salinity and temperature history of the deglaciation at the mouth of the St Lawrence drainage system, with special attention to the Younger Dryas interval. We used three cores raised, respectively, from the central Cabot Strait area, the Laurentian Channel of the Scotian shelf, and the Laurentian Fan, about 30 km off the shelf break (Fig. 1).

Modern fresh water from the St Lawrence drainage basin mixes with the North Atlantic water masses in the study area^{11,12} (Fig. 1). The coring sites thus have a strategic location for assessing the impact of meltwater pulses through the St Lawrence outlet. Core 89-007-111 was raised from Cabot Strait, which constitutes the 'bottleneck' for freshwater and/or meltwater discharges into the ocean. An increasing salinity gradient is then observed offshore, from the continental shelf (core 90-031-047) to the slope (core 90-031-044). The freshwater outflow (annual mean of 0.018 Sv; ref. 12) is at present responsible for a buoyant low-saline surface

TABLE 1 Radiocarbon dates

Depth (cm)	Material dated	Corrected ^{14}C age (yr)	IsoTrace lab. no.
Core 89-007-111P			
68–69	<i>Scaphander</i> sp.	5,990	TO-1875
115–117	<i>Yoldia</i> sp.	7,820	TO-1876
325–326	<i>Nuculana</i> sp.	10,200	TO-1877
590–592	Pelecypod	11,690	TO-1878
Core 90-031-044P			
19.3–21.3	Npl	2,210	TO-4004
39–41	Npl	3,830	TO-4005
139–141	Npl	9,640	TO-4006
179–181	Npl	10,100	TO-4007
219–221	Npl	10,440	TO-4008
299–301	Npl	11,390	TO-4409
409–411	Npl	12,490	TO-4606
549–551	Npl	12,550	TO-4607

Ages were determined by accelerator mass spectrometry at IsoTrace, Toronto and were calculated after isotopic normalization (for a $\delta^{13}\text{C}$ value of -25‰) using Libby's ^{14}C half-life. The ages were also corrected by -400 years to account for the apparent age of dissolved inorganic carbon in high-latitude surface waters of the North Atlantic²⁷.

layer (0 to about 50 m; ref. 13) characterized by a salinity gradient ranging from 30 to 32‰ in summer, from Cabot Strait to the shelf break (Fig. 1). This surface water mass overlies an intermediate (~ 50 – 200 m) cold-water layer fed by the Labrador current, partly mixed with a deeper, relatively warm and saline North Atlantic water mass¹³.

Oxygen isotope stratigraphies were established in the shelf cores 89-007-111 and 90-031-047, based on analyses of planktic *Neogloboquadrina pachyderma* (left-coiling; Npl) and benthic *Elphidium excavatum* foraminifers. In both cases, the sparse planktic populations exclusively dominated by Npl yielded discontinuous isotope records (Fig. 2). A few ^{14}C ages were also obtained from accelerator mass spectrometry (AMS) measurements on gastropod shells in core 89-007-111. In the slope core 90-031-044, continuous ^{18}O measurements and seriated AMS ^{14}C ages were obtained for Npl assemblages (Table 1). ^{14}C stratigraphies indicate fairly high apparent sedimentation rates during the deglaciation, of about 90 and 170 cm kyr^{-1} in cores 90-031-044 and 89-007-111, respectively. With analyses performed at 10-cm intervals (that is, at intervals thicker than one biologically mixed layer¹⁴), the time resolution ranges between fifty and one hundred ^{14}C years and seems suitable for determining hydrographic changes at the timescale of Younger Dryas event.

The isotope stratigraphies in the three cores are consistent on a regional scale. In core 90-031-047, similar ^{18}O contents in planktic and benthic foraminifers are observed, suggesting that the isotope signature of Npl relates to a relatively deep mesopelagic water mass, rather than to the surface-water layer (see also ref. 15). It is notable that the isotope record does not show any significant shift which may be attributed to a dilution with a major meltwater pulse within the Younger Dryas time frame. In view of correlations with a reference record for the southwestern Labrador Sea (core 91-045-094; Fig. 1; ref. 16), it seems likely that Npl assemblages in the Laurentian Channel and Fan cores rather bear an isotope imprint of the sub-surface Labrador current flowing southwards along the Canadian coast. The stratigraphy of the studied cores is further constrained by pollen data allowing a direct correlation with the on-shore palynostratigraphy (Fig. 2; refs 17–19). In addition, dinoflagellate cyst assemblages are characterized by sharp changes enabling us to establish a regional ecostratigraphy¹⁷. This ecostratigraphy primarily reflects cold surface waters throughout ecozone I, that is before 10,000 BP, with particularly harsh conditions corresponding to subzone Ib (Fig. 2). Ecozone II corresponds to improving sea-surface conditions evolving towards the modern situation¹⁷.

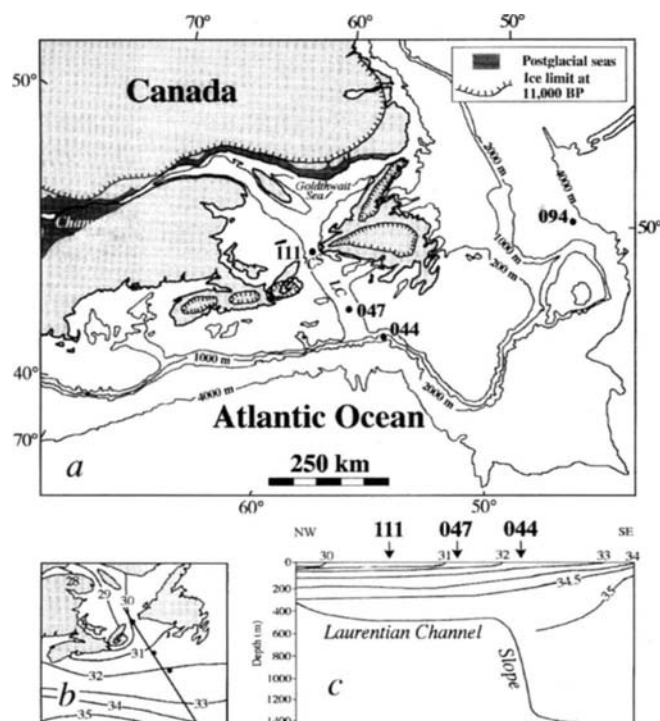
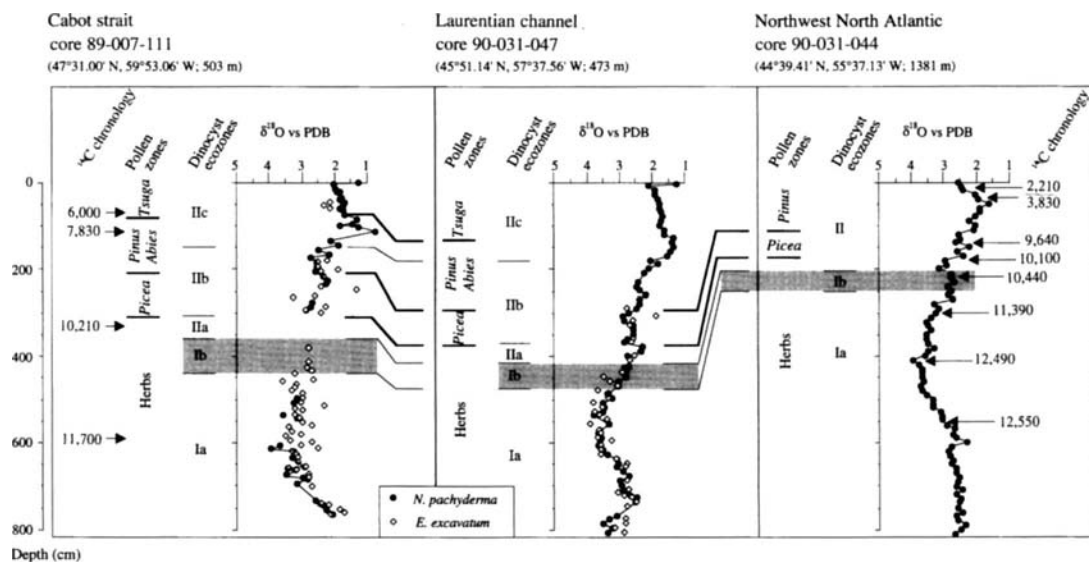


FIG. 1 a, Coring site location. CS, Cabot Strait; LC, Laurentian channel. Core numbers are abbreviated as follows: 111, core 89-007-111 (see ref. 17 for details on the stratigraphy); 047, core 90-031-047; 044, 90-031-044; 094, 91-045-094 (see ref. 16 for detail on the stratigraphy). b, Distribution of salinity in surface water (compiled from the data set of the World Ocean Atlas¹⁴). Dots correspond to coring site locations; the line corresponds to the vertical salinity profile in c. c, Vertical distribution of salinity along the outlet of the St Lawrence drainage basin, from the Gulf of St Lawrence to the North Atlantic (arrows indicate the respective locations of coring sites)¹¹.

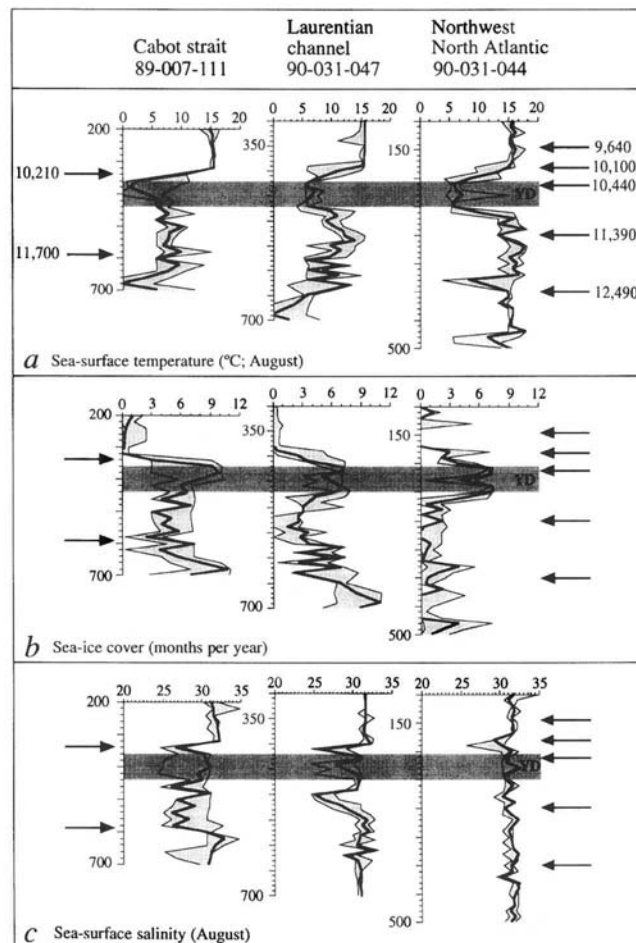
FIG. 2 Chronostratigraphy, isotope record and eco-stratigraphy of cores 89-007-111, 90-031-047 and 90-031-044. The pollen zonation¹⁷ is established after direct correlations with the onshore palynostratigraphy of the Atlantic provinces of Canada (see, for example, ref. 19). The lower 'herbs' zone corresponds to an assemblage with abundant non-arboreal taxa (Asteraceae, Cyperaceae, Gramineae, *Alnus*, *Salix*, *Betula*, Ericaceae); it is associated with a tundra-type vegetation over Canada. The 'Picea' zone corresponds to the regional afforestation after ~10,000 BP. High *Pinus* percentages and significant occurrence of *Abies* ('*Pinus-Abies*' zone) is regionally recorded after about 9,000 BP. Finally, the significant occurrence of *Tsuga* on a regional scale constitutes a time mark at ~6,000 BP. The dinocyst zonation is defined after correlations on a regional scale¹⁷. Ecozone I is characterized by dominant Peridinales (*Brigantidium*, *Algidasphaeridium minutum* and *Peridinium faeroense*), which are almost exclusive in subzone Ib. Ecozone II is marked by abundant Gonyaulacales and diversification of flora, with the successive occurrences of *Bitectatodinium tepikiense* and *Alexandrium excavatum* (zone I/II



transition), *Spiniferites ramosus* (subzone II1-b transition) and *Nematosphaeropsis labyrinthus* (subzone IIb-c transition). Details of the regional ecostratigraphy (including core 89-007-111) are published elsewhere¹⁷. Shaded zone corresponds to ecozone Ib, which is marked by particularly cold conditions and associated with the Younger Dryas event. In cores 89-007-111 and 90-031-044, it is dated between 10,800 and 10,300 BP.

Sea-surface conditions can be quantitatively estimated using transfer functions based on the dinoflagellate cyst assemblages. In contrast to planktic foraminifers, dinoflagellates do occupy the photic zone, their cysts, which are related to sexual reproduction, yield assemblages allowing the reconstruction of sea-surface conditions such as temperature and salinity (in February and August), in addition to the seasonal extent of sea-ice cover^{3,4}. The use of the best analogue method²⁰ provides accurate reconstructions (that is, within the range of interannual variability). The reference database, which includes a large number of sites in neritic

FIG. 3 Estimates of sea-surface temperature in August (a), seasonal duration of the sea-ice cover (b) and sea-surface salinity in August (c). The sea-ice cover corresponds to the number of months per year with more than 50% of sea-ice coverage. Thick lines indicate the most probable values (weighted means of environmental values for the ten closest modern analogues); thin lines delimit the confidence interval that corresponds to the minimum and maximum statistically possible according to the set of 10 analogues (for details on the method see refs 3, 4 and 20). Note that estimates of temperature, sea-ice cover and salinity are reported versus depth (in cm) in the cores for the interval of interest. The ¹⁴C chronology is indicated by arrows (compare Table 1). The grey zone is the same as in Fig. 2. It corresponds to the coldest phase of the Younger Dryas (YD) which is recorded between 10,800 and 10,300 BP. The reconstructions rely on an updated reference database including 438 sites from the middle to high latitudes of the North Atlantic and adjacent basins. Environmental parameters were compiled from data sets provided by Environment-Canada, the National Climate Data Center²⁸, and the National Oceanographic Data Center¹¹. The last validation runs (July 1995) on the updated data base yielded accurate results: coefficients of correlations between estimates and instrumental values are greater than 0.93 for all parameters, with more than 95% of reconstructions included within one standard deviation around instrumental data averages. For what concerns the salinity, for example, the standard deviation of the difference between reconstructions and the mean instrumental average is 0.7‰, which also corresponds to the average standard deviation around the mean for instrumental values.



environments, allows the reconstruction of salinities ranging from 25 to 36‰, with an overall precision of ~0.7‰ (Fig. 3 legend).

The late glacial interval which corresponds to ecozone I (>10,000 BP) was characterized by changes in sea-surface conditions of relatively large amplitude (Fig. 3). Offshore (cores 90-031-047 and 90-031-044), sea-surface temperatures fluctuated between 5 and 16 °C in August, and sea-ice cover varied from 0 to 7 months of the year. In Cabot Strait area (core 89-007-111), late glacial sea-surface temperatures varied from 0 to 10 °C, pointing towards colder conditions that probably arose in response to the direct impact of ice melt water and to the proximal influence of continental ice masses. Despite regional differences in the late glacial sea-surface temperature ranges, all cores indicate particularly harsh conditions in the top of ecozone I (subzone Ib), dated at ~10,800 to 10,300 BP and corresponding to the regional signature of the Younger Dryas. During the Younger Dryas, cold summer temperatures (1 to 5 °C in August) and extensive sea-ice cover for about 7 to 10 months of the year prevailed on a regional scale (see also ref. 17).

The late glacial part of the records from the Cabot Strait and the Laurentian channel shows fluctuations in sea-surface salinity which indicate meltwater pulses through the St Lawrence pathway. In Cabot Strait (core 89-007-111), an initial lowering of salinity is recorded before 11,700 BP (see also ref. 21). It corresponds to the Champlain Sea submergence of the St Lawrence lowlands^{21,22}. During the Champlain Sea episode, spanning about 11,700 to 10,000 BP, surface salinities at the outlet of the Gulf of St Lawrence remained generally low, as a result of ice meltwater outflow through the Champlain Sea and the Goldthwait Sea, then occupying the Gulf of St Lawrence²¹. However, the influence of meltwater runoff was apparently restricted to the uppermost water layer because it did not affect the mesopelagic or bottom-water layers of the Laurentian channel, as indicated by both the isotope stratigraphy (Fig. 2) and the benthic foraminifer assemblages²¹. Moreover, about 30 km off the shelf break in the north-west North Atlantic (core 90-031-044), there is no evidence for any significant salinity anomaly in surface waters, at least within the level of accuracy of the method (~0.7‰), before and during the Younger Dryas event. Sea-surface salinities remained close to modern values (32‰ on the average) above the continental slope throughout the deglaciation. The meltwater runoff and glacial lake drainage waters (for example, Lake Agassiz) probably vanished in the neritic domain before reaching the North Atlantic and mixing with open ocean waters. Depending on the rate of open ocean mixing and on the sensitivity of the thermohaline circulation (see ref. 23, for example), the absence of significant salinity changes off the shelf break suggests that the Younger Dryas was not triggered by a sudden salinity dilution in the North Atlantic resulting from the diversion of the Laurentide ice melt water from the Mississippi to the St Lawrence drainage system, as proposed by Broecker *et al.*².

During the Younger Dryas interval, an increase in salinity is shown by both shelf records, despite extensive sea-ice cover (Fig. 3) which is responsible for diluting surface water during summer. Sea-surface salinities were similar to modern ones, suggesting that the total freshwater flux from the St Lawrence pathway was of the same order of magnitude, and probably less than today, during the Younger Dryas. In fact, the increase in salinity at the mouth of the St Lawrence pathway during the Younger Dryas rather indicates significantly reduced meltwater runoff from the southern Laurentide ice margin then stabilized on the St Narcisse end moraine position extending over 500 km, north of the St Lawrence lowland and estuary²⁴⁻²⁶. □

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Early Carboniferous tetrapods in Australia

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THE earliest tetrapod fauna yet discovered in the Southern Hemisphere comes from the Drummond basin of central Queensland, Australia. The fauna is of Early Carboniferous age (mid-Viséan, about 333 Myr ago¹) and comprises at least three types of tetrapods in association with abundant freshwater fishes. Two of the tetrapods (a colosteid and, less certainly, a temnospondyl) are distantly related to living amphibians, whereas the third (an anthracosaur) is an early relative of the amniotes. All three tetrapods rank among the oldest known representatives of their clades, and two of them (colosteid and anthracosaur) represent groups previously confined to Europe and North America. By virtue of its geographic remoteness, early age, and taxonomic diversity, the Drummond basin fauna has important implications for current understanding of early tetrapod history^{2,3}. Most significantly, it indicates that several major groups of tetrapods were distributed worldwide through equatorial regions during the Early Carboniferous.

The fauna was discovered in mudstones of the Ducabrook Formation⁴ at a locality named Middle Paddock, in the southern part of the Drummond basin, east central Queensland (Queensland Museum field locality L1117). The Ducabrook Formation is a thick (>2,100 m) sequence of interbedded sandstones and mudstones, predominantly khaki brown and olive green in colour, with occasional beds of conglomerate, tuff, limestone and algal

nodules. These sediments probably accumulated in shallow fluvial and lacustrine environments⁴, and have previously yielded palaeoniscoids (*Elonichthys*)⁵, acanthodians (*Gyracanthides*)⁶, branchiopods (*Leaia*)⁴, plant macrofossils⁴ and a rich palynoflora⁷ of mid-Viséan age (Fig. 1).

Newly collected material is dominated by remains of freshwater fishes, principally acanthodian finspines (*Gyracanthides* cf. *G. murrayi*), with occasional fragments provisionally attributed to rhizodonts and lungfishes. Isolated tetrapod bones are reasonably common, well preserved and uncrushed, although many are broken and slightly weathered. All specimens are registered at the Queensland Museum, Brisbane.

Several cranial elements, including premaxilla (Fig. 3a–d), maxilla (Fig. 4a) and ectopterygoid (Fig. 4b), represent a tetrapod

of the family Colosteidae (*sensu* Hook⁸), widely regarded as the sister-group of the Temnospondyli or of the Temnospondyli plus Microsauria^{3,9} (Fig. 2). The premaxilla shows diagnostic colosteid features, including a single tusk incorporated in the marginal tooth row, but offset medially from it, and a palatal ramus contributing to the rim of the choana (Fig. 3e). Its broken tusk reveals the distinctive histological pattern defined¹⁰ as labyrinthodont *sensu stricto*, but with the addition of marginal wedges of 'dark dentine'. Although this overall pattern of tooth structure, including 'dark dentine', is best documented in anthracosaurs¹¹, it may be common among early tetrapods¹² (excluding temnospondyls¹³), and we suspect that it will prove to be shared by other colosteids. The Middle Paddock colosteid is one of the earliest yet reported, rivaling in age *Pholidogaster* from the Viséan of Scotland¹⁴, and we believe it to be the first discovered outside Britain or North America.

Several quadrate bones (Fig. 4c) show surprising resemblance to those of Triassic temnospondyls, particularly in their screw-shaped condylar region with its anteriorly extended medial condyle. Although the condylar surface is poorly known among early temnospondyls, it appears to have been slightly screw-shaped in *Balanerpeton* (Viséan)⁹, and is markedly so in *Trimerorhachis* (Early Permian; American Museum of Natural History, specimen 4763) and in the loxommatid *Megaloccephalus* (Late Carboniferous)¹⁵. The quadrates of anthracosaurs and colosteids (known only in *Greerpeton*¹⁶) are quite different, with the medial condyle forming a narrow transverse roller alongside a much smaller and poorly differentiated lateral condyle. If a temnospondyl is present in the Middle Paddock fauna, it would be one of the earliest on record, being roughly contemporaneous with *Balanerpeton* from the Viséan of Scotland⁹. Such an occurrence would be consistent with the unparalleled diversity subsequently attained by temnospondyls in East Gondwana¹⁷. It seems less likely that the Middle Paddock quadrate bones should represent loxommatids, which appear to have been confined to Euramerica.

The presence of an anthracosauroid tetrapod (suborder Anthracosauroida, *sensu* Smithson¹⁸) is indicated by a near-complete ilium with robust postiliac process (Fig. 4d). The postiliac process is well developed in primitive tetrapods^{19,20} and anthracosaurs²¹, but is not found in colosteids or temnospondyls, where it is reduced to a short spur^{21,22}. In view of its Viséan age, the ilium from Middle Paddock seems likely to represent an anthracosaur rather than some less-derived tetrapod resembling *Ichthyostega* or *Acanthostega*. This identification may be supported by additional fragments of dermal bone with ornament unlike that of colosteids or temnospondyls but similar to the ill-defined pitting characteristic of anthracosaurs^{11,21}. If an anthracosaur is present at

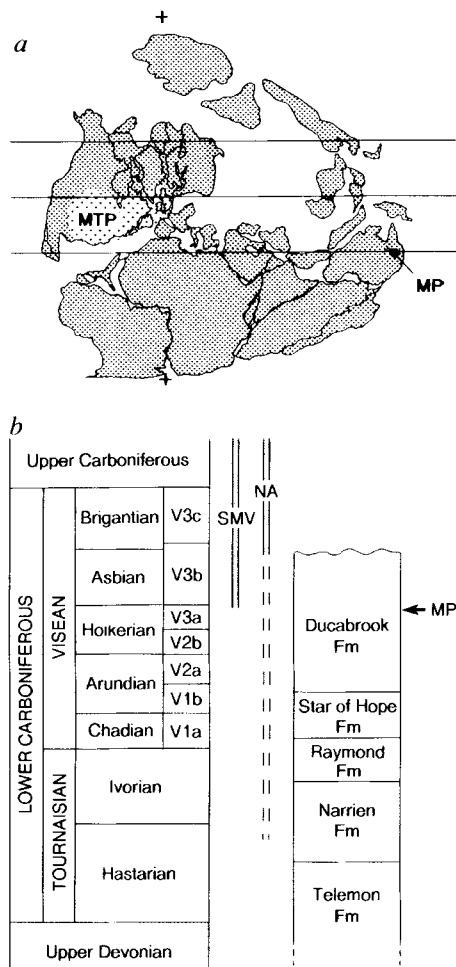


FIG. 1 Stratigraphic and geographic context of Viséan tetrapods of the Drummond basin. a, Palaeogeographic map showing distribution of Viséan tetrapods; horizontal lines indicate Early Carboniferous equator and tropics; MP, Middle Paddock site; MTP, Mississippian Tetrapod Province², encompassing all other sites for Early Carboniferous tetrapods. b, Stratigraphic columns showing Drummond basin sequence in relation to tetrapod-bearing horizons of Euramerica. Vertical bars indicate known stratigraphic ranges for Early Carboniferous tetrapods of North America (NA) and the Scottish Midland Valley (SMV). The North American range is extended to include the Tournaisian Horton Bluff Formation of Nova Scotia, which has yielded a few undescribed tetrapod fragments. Stratigraphic level of the Middle Paddock fauna indicated by an arrow (MP). Microvertebrate assemblages²⁹ indicate that the Upper Telemon Formation is Early Tournaisian in age, and that the Raymond Formation is Late Tournaisian or Early Viséan. Deposition of the Drummond basin sediments ceased before the end of the Viséan⁷; along the southwestern margin of the basin the Ducabrook Formation is overlain disconformably by the Lake Galilee Sandstone, dated as Namurian A on the evidence of palynology³⁰.

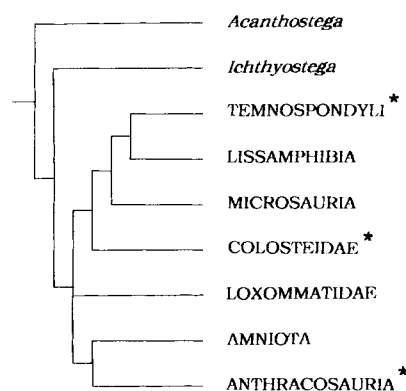


FIG. 2 Relationships of tetrapod clades mentioned in text (adapted from ref. 3). Lissamphibia, comprising modern amphibians, is probably a clade nested within Temnospondyli, but is shown as its sister-group for the sake of simplicity. Asterisks indicate clades represented in the Middle Paddock fauna.

the Middle Paddock site it is the first example discovered outside Euramerica, and is at least as old as the earliest known forms, *Silvanerpeton*²³ and *Eldeceon*²⁴, both from the Viséan of Scotland. Alternatively, the ilium (Fig. 4d) could represent a problematical tetrapod such as *Crassigyrinus*¹¹ (Viséan-Namurian of Scotland), which may be a primitive stem tetrapod convergently resembling anthracosaurs^{3,12}.

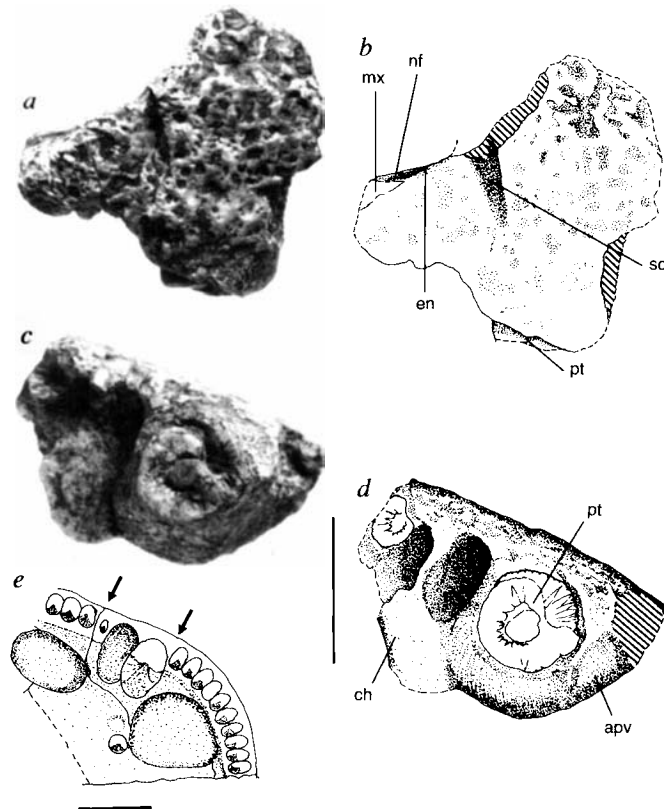


FIG. 3 Right premaxilla of colosteid (QM F34281) from Middle Paddock site. a, c, Lateral and palatal views. b, d, Corresponding diagrammatic keys. e, Comparative palatal view of right premaxilla in North American colosteid *Greererpeton burkemorani* (after Smithson¹⁶); arrows indicate region preserved in Middle Paddock specimen. Pitted ornament characteristic of colosteids¹¹ is obscured by weathering but is better preserved on other skull fragments (for example, the maxilla in Fig. 4a). The premaxilla differs from that of *Greererpeton* in several details: the tusk is circular in cross-section, rather than elliptical, and there are two tooth sites behind the tusk pair (the tusk and replacement pit), whereas *Greererpeton* has only one¹⁶. A small facet on the subnarial ramus (mx) indicates that maxilla overlapped premaxilla; the reverse is seen in *Greererpeton*¹⁶. A steeply inclined groove (sc) immediately in front of the external naris probably accommodated a sensory canal; in other colosteids the equivalent groove, where identifiable^{8,16}, is more widely separated from the naris. The broken surface of the premaxillary tusk (pt) reveals a distinctive histological pattern defined¹⁰ as labyrinthodont *sensu stricto*. This comprises tightly packed folds of plicidentine, without secondary folds or branches, surrounding a pulp cavity void of osteodentine; bone of attachment does not intervene between the external folds of the tusk. The broken surface also reveals marginal wedges of 'dark dentine', a tissue identified in anthracosaurs¹¹ but not in temnospondyls¹³, that is defined not by intensity of coloration, which reflects vagaries of preservation, but by dense packing of dentine tubules with radial orientation¹⁰; here the 'dark dentine' is lighter in colour than the intervening folds of plicidentine, as in some other early tetrapods¹¹. Abbreviations: apv, steep rear wall of anterior palatal vacuity; ch, rim of choana; en, anteroventral rim of external naris; mx, sutural contact with maxilla; nf, floor of narial chamber; pt, premaxillary tusk; sc, sensory canal. Scale bars, 10 mm.

Despite its remote geographic location, the Middle Paddock fauna bears striking resemblance to Euramerican faunas that have been united² under the rubric of the Mississippian Tetrapod Province (MTP; Fig. 1a). The resemblance extends, for example, to the Late Viséan fauna of Delta, Iowa, which was located virtually at the opposite pole of the Early Carboniferous globe, yet shared a similar association of *Gyracanthus*, palaeoniscoids, rhizodonts, dipnoans, a colosteid and a 'proto-anthracosaur' (*Whatcheeria*)^{25,26}. Evidently the MTP was not confined to the southern margin of Euramerica² but may have extended through a tropical belt across the whole of Pangaea. This immense geographic range implies that the northwestern margin of Gondwana had approached, or collided with, the southern margin of Euramerica before the mid-Viséan, thereby opening a route for the trans-Pangaean dispersal of continental vertebrates.

Hitherto the entire fossil record of Devonian and Early Carboniferous tetrapods in Gondwana comprised a single jaw of very primitive appearance²⁷, and two occurrences of fossil footprints^{17,28}, all from the Devonian of Australia. Although this meagre evidence hinted that tetrapods might have originated in East Gondwana, a detailed understanding of early tetrapod history was inevitably based on the more substantial fossil record of Europe and North America^{2,3}. In these circumstances the Middle Paddock site is important in providing the first glimpse into the composition of an early tetrapod fauna outside Euramerica. It reveals that a remarkably consistent suite of continental

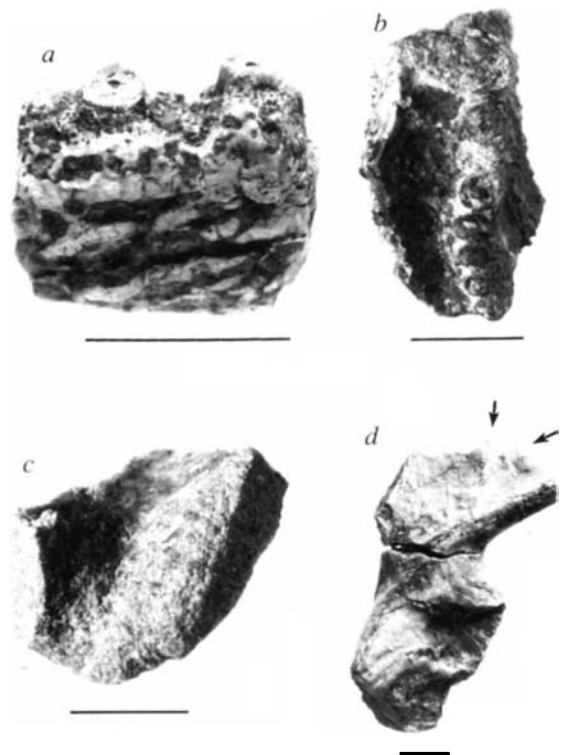


FIG. 4 Viséan tetrapod bones from Middle Paddock site. a, Colosteid maxillary fragment (QM F34285) in lateral view. b, Left ectopterygoid of colosteid (QM F34282) in palatal view, showing the stump of a large tusk followed by a slightly curved line of smaller teeth, which decrease in size posteriorly. Medial to this tooth row the palatal surface shows pronounced downwards curvature towards the pterygoid; this degree of curvature is unrivalled in other colosteids, where it might perhaps have been masked by crushing²⁶. c, Right quadrate provisionally attributed to ?temnospondyl (QM F34284) in palatal view. d, Left ilium of anthracosauroid (QM F34280) in ventrolateral view; a distinct crescentic notch is preserved intact at the dorsal margin (between arrows), separating the broken base of the robust postiliac process (upper right) from the thinner iliac blade (upper left). Scale bars, 10 mm.

vertebrates, including a variety of fishes and several major groups of tetrapods, was distributed worldwide through equatorial regions by the Early Carboniferous. □

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Anatolian tree rings and the absolute chronology of the eastern Mediterranean, 2220–718 BC

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EXCELLENT preservation of wood and charcoal at archaeological sites in Anatolia has allowed the Aegean Dendrochronology Project to build absolute and floating tree-ring sequences¹. One such floating dendrochronology of 1,503 years includes samples relating to known rulers, sites and cultures of the ancient eastern Mediterranean. If this chronology could be dated precisely, many long-standing questions might be resolved. Here we report 18 high-precision ¹⁴C determinations which, when wiggle-matched to the radiocarbon calibration curve, provide a date within narrow limits. Inside this range, we can suggest the probable

absolute dating of the dendrochronology because of a remarkable growth anomaly in the seventeenth century BC, for which we propose a correlation with major growth anomalies at 1628/1627 BC in the absolutely dated dendrochronologies of Europe and the United States. Many archaeological sites from several cultures in the eastern Mediterranean can now be dated with fine precision. This chronology has important implications for Old World archaeology and prehistory.

A tree-ring chronology of 1,503 years has been constructed from large groups of timbers in major archaeological monuments, principally at Gordion, Porsuk, Acemhöyük and Kültepe in Anatolia², and might provide a solution to many currently impenetrable or ambiguous issues in eastern Mediterranean archaeology. However, this prehistoric chronology is floating, that is, it is not connected at present to a fixed dendrochronology from living trees backwards.

We have dated this chronology by obtaining high-precision ¹⁴C determinations on a sequence of decadal samples from it, and matching the results with the precisely dated decadal variations in radiocarbon ages known for European wood (Table 1; Figs 1 and 2). A placement of the end of the 350-year radiocarbon-dated sequence at ~820 BC is most likely (with an estimated 2σ, 95.4%, confidence error of +76/–22 calendar years). The entire 1,503-year dendrochronology² thus begins at 2233 BC and runs to 731 BC (+76/–22 years).

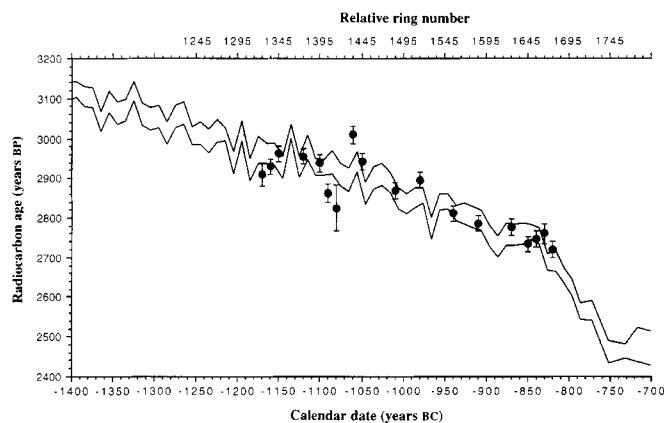


FIG. 1 Wiggle-match (best fit) of the 18 dendrochronologically sequenced ¹⁴C measurements (Heidelberg data) (1σ error bars shown) against the 1993 decadal radiocarbon calibration curve (1σ confidence band)²⁵. The 350-year dated sequence (relative rings 1325 to 1675) begins at 1170 BC and ends at 820 BC. Estimation of the error on the above fit is problematic. It should include: (1) the combination of the measurement errors on the Heidelberg data and the calibration curve (18 paired measurements, which yields ±8 ¹⁴C years); (2) the likely systematic laboratory offset between Heidelberg and the relevant calibration laboratory (Seattle), estimated as ± ≤ 20 ¹⁴C years (from intercalibration of identical wood samples between the Seattle and Heidelberg laboratories; M. Stuiver, personal communication; B. Kromer, personal communication); (3) the error from the potential mismatching of the specific decades dated at Heidelberg versus those dated for the calibration curve (estimated as ± ≤ 15 ¹⁴C years, from analysis of ref. 25); and (4) the possibility of small regional differences in ¹⁴C levels^{27,28}, which between central European oak and central Anatolian juniper we estimate as ± ≤ 15 ¹⁴C years. (This is a highly speculative issue which we address by specifically using calibration data based on wood (central Europe) growing nearest to Anatolia, and without major ocean input or extreme altitude difference. Further, even in the claimed cases^{27,28} of differences between sequoia from the west coast of the United States and Irish oak, and Irish oak and German oak, a dominant contribution from laboratory offsets and measurement errors cannot be excluded.) Therefore an overall combined error of ± ≤ 30 ¹⁴C years exists on our wiggle-match at 1σ confidence. Conversion of this ¹⁴C-year confidence interval onto the calendar scale is asymmetrical, as the shape of the radiocarbon calibration curve constrains the lower limit much more than the upper one. We estimate the fit as +76/–22 calendar years at 2σ, 95.4%, confidence.

TABLE 1 Radiocarbon dates for 10-year samples of juniper

Relative ring/year	^{14}C age (BP $\pm \sigma$)	χ^2 best fit, date (BC)	OxCal 68.2% confidence date (BC)	OxCal 95.4% confidence date (BC)
1675	2720 $\pm$ 20	820	818–826	814–840 ($P = 0.86$), 847–853 ($P = 0.14$)
1665	2760 $\pm$ 25	830	828–836	824–850 ($P = 0.86$), 857–863 ($P = 0.14$)
1655	2746 $\pm$ 20	840	838–846	834–860 ($P = 0.86$), 867–873 ($P = 0.14$)
1645	2734 $\pm$ 20	850	848–856	844–870 ($P = 0.86$), 877–883 ($P = 0.14$)
1625	2777 $\pm$ 20	870	868–876	864–890 ($P = 0.86$), 897–903 ($P = 0.14$)
1585	2786 $\pm$ 20	910	908–916	904–930 ($P = 0.86$), 937–943 ($P = 0.14$)
1555	2811 $\pm$ 20	940	938–946	934–960 ($P = 0.86$), 967–973 ($P = 0.14$)
1515	2895 $\pm$ 20	980	978–986	974–1000 ($P = 0.86$), 1007–1013 ($P = 0.14$)
1485	2868 $\pm$ 20	1010	1008–1016	1004–1030 ($P = 0.86$), 1037–1043 ($P = 0.14$)
1445	2942 $\pm$ 21	1050	1048–1056	1044–1070 ($P = 0.86$), 1077–1083 ($P = 0.14$)
1435	3009 $\pm$ 23	1060	1058–1066	1054–1080 ($P = 0.86$), 1087–1093 ($P = 0.14$)
1415	2825 $\pm$ 59	1080	1078–1086	1074–1100 ($P = 0.86$), 1107–1113 ($P = 0.14$)
1405	2863 $\pm$ 23	1090	1088–1096	1084–1110 ($P = 0.86$), 1117–1123 ($P = 0.14$)
1395	2938 $\pm$ 23	1100	1098–1106	1094–1120 ($P = 0.86$), 1127–1133 ($P = 0.14$)
1375	2955 $\pm$ 20	1120	1118–1126	1114–1140 ($P = 0.86$), 1147–1153 ($P = 0.14$)
1345	2962 $\pm$ 20	1150	1148–1156	1144–1170 ($P = 0.86$), 1177–1183 ($P = 0.14$)
1335	2929 $\pm$ 20	1160	1158–1166	1154–1180 ($P = 0.86$), 1187–1193 ($P = 0.14$)
1325	2909 $\pm$ 28	1170	1168–1176	1164–1190 ($P = 0.86$), 1197–1203 ($P = 0.14$)

^{14}C measurements (18) of 10-year samples of juniper from one log, crossdated with 40 other timbers for its entire length, from the Midas Mound tumulus at Gordion (Heidelberg data). The samples are from relative rings/years 1320–1330 to 1670–1680 (the whole 1,503-year chronology runs from relative rings/years 262 to 1764); the ^{14}C determinations are regarded as belonging to the midpoint of each dated decade. The sequence of data was then matched to the 1993 decadal radiocarbon calibration curve²⁵. This data set was selected because: (1) its resolution most closely corresponds to that of our data; (2) it reflects the most accurate estimate of atmospheric radiocarbon variations in the age range of our chronology; and (3) the data are from trees that grew in central Europe, so they are the most compatible and representative available for wood from Turkey. The data were best-fitted to the calibration curve by using two computer programs: a chi-squared, least-squares difference, procedure²¹, and a probability procedure (OxCal 2.18)²⁶. From the former, at 95.4% confidence, the midpoint of the most recent dated decade belongs between 894 BC and 789 BC. More specifically, the best fit is at 820 BC (see Fig. 2). The OxCal program suggested that four measurements were potentially inconsistent based on quoted errors (at rings 1325, 1405, 1415 and 1435), with the data for rings 1405 and 1435 less consistent than rings 1325 and 1415. If all 18 data are used, the OxCal 95.4% confidence range for the end date of the sequence is 814 BC to 840 BC ($P = 0.86$) and 847 BC to 853 BC ($P = 0.14$) (at 68.2% confidence: 818 BC to 826 BC). If only the best 16 data are used: 816 BC to 842 BC ($P = 0.94$) and 847 BC to 853 BC ($P = 0.06$) (at 68.2% confidence: 821 BC to 829 BC). If the best 14 data are used: 815 BC to 875 BC (at 68.2% confidence: 820 BC to 829 BC ($P = 0.64$) and 846 BC to 850 BC ($P = 0.36$)).

Within the dating 'window' established independently by the radiocarbon evidence, we believe we can provide an even more precise date for the dendrochronology. In 36 trees from the site of Porsuk in south-central Anatolia³, with tree rings dated to the 17th century BC by the radiocarbon wiggle-match, there is an exceptional growth event at and immediately following relative ring 854 ($\sim 1,641 \pm 76/-22$ BC from the wiggle-match) (Fig. 3). In dendrochronologies from Europe and the United States, a similar dramatic, cooler, wetter climatic event is also recorded in the 17th century BC, absolutely dated at 1628/1627 BC^{4,5}. As in the Anatolian tree-ring chronology, this event is unusual, and is the only such event in the 18th–15th centuries BC. It correlates

with evidence from Greenland ice cores of a major volcanic eruption and other data reflecting a major climatic/atmospheric anomaly^{5,6}. The year 1628 BC can be regarded as a special marker event in Northern Hemisphere tree-ring chronologies⁵.

We propose that the Anatolian ring 854 event be correlated with the events of 1628/1627 BC in Europe and the USA, because it is compatible with the date range established by the radiocarbon wiggle-match. We find support from a second coincidence: 470 years after the ring 854 event, another growth anomaly (significant but much smaller than that of the 17th century BC) begins at ring 1324 in the Anatolian dendrochronology⁷. This provides a very good match ($\pm 0-1$ year) with the major anomaly commencing in

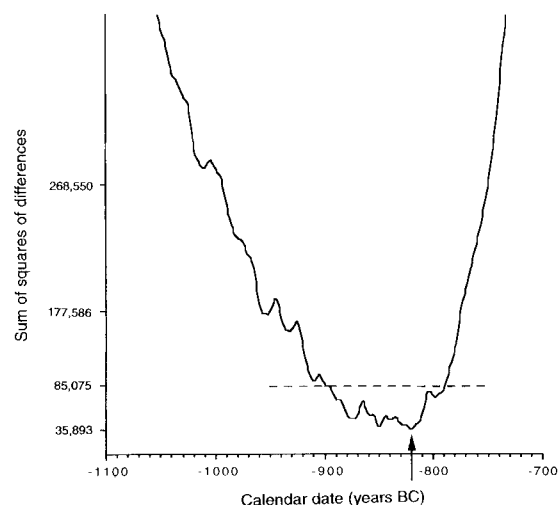


FIG. 2 The chi-squared fit function (based on sum of squares of differences) for the wiggle-match of the 18 dendrochronologically sequenced ^{14}C measurements (Heidelberg data) against the 1993 decadal calibration curve²⁵ (cubic spline through data points to produce an annual curve) shown in terms of the fit of the end date for the 350-year dated sequence (decades from relative rings 1320–1330 to 1670–1680; date regarded as the midpoint of each decade). The best fit is at 820 BC (arrow; $\chi^2 = 12.36$) (and generally $\pm$ about two decades). A fit around 850 BC is also possible, but less likely. The OxCal 2.18 data (see Table 1) reflect this same potential ambiguity: a region around 820 BC is most likely, although a lesser possibility exists around 850 BC. The fit is good, but by no means perfect. It would be desirable to date down the steep slope in the radiocarbon calibration curve 820 BC–750 BC, but there is insufficient sample material for high-precision dating from the Anatolian dendrochronology. However, a perfect fit should not be expected: even the best high-precision radiocarbon laboratories exhibit small systematic offsets from each other^{27,29}. The broken line indicates a 95% significance level; $\chi^2 = 27.59$, 17 d.f.; the fit is therefore between 894 BC and 789 BC.

1159 BC known from Europe⁵. This is the only such important event for several centuries in either chronology. This 1159 BC event is linked to the eruption of Hekla 3 in Iceland, as attested in ice-core data from Greenland⁵. The very high latitude of this volcano, and the strong poleward transport of atmospheric circulation, explains the reduced impact in Anatolia and the lack of evidence for a large effect (frost damage) in the White Mountains of California.

The combination of high-precision radiocarbon wiggle-matching and the correlation of dendro-marker events offers a likely absolute date for the 1,503-year chronology from Anatolia. The anomalies from the 17th and 12th centuries BC probably result from large, climatically effective, volcanic eruptions, the resultant aerosol forcing atmospheric cooling for a few years^{4,5}. Evidence for suitable, large volcanic eruptions is found at these times in Greenland ice cores^{5,8,9}. Correlation of the few major common events in the tree-ring and ice-core chronologies allows the specific identification of an important volcanic event at 1628 BC^{5,6,10,11}.

The exceptional growth of relative ring 854 is continuous in both early and late wood, so the responsible climate-modifying event (the volcanic eruption) must have occurred immediately before the beginning of spring growth. The event/eruption shows up in the spring of 1628 BC in Ireland and then, as temperatures in the northern hemisphere drop as a result of aerosol forcing, as frost damage in 1627 BC in California at the upper timber line. An eruption date in late 1629 BC (after the growing season) or early 1628 BC is likely. Thus ring 854 of our 1,503-year chronology relates to 1628 BC.

This new chronology is of wide significance. A nexus of buildings, sites, personages and events may now be directly and precisely dated. The chronology dates 22 major sites, ending

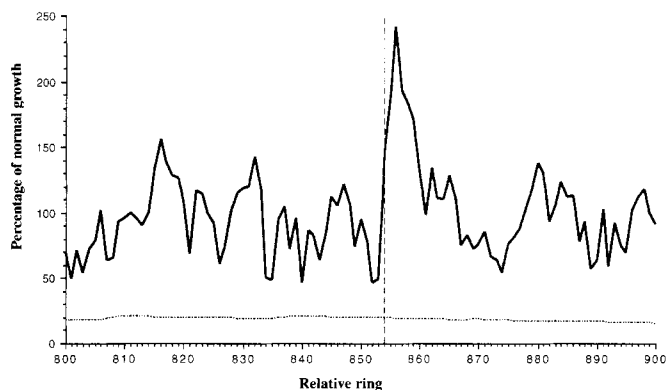


FIG. 3 In relative year 854 (shown by the vertical dashed line) a population of juniper trees (number of samples shown by dotted line, variously 15–21) ranging in age (in relative ring/year 854) from 19 to at least 143 years experiences a sudden growth event. This is also reflected in cedar and pine trees from the site (total number of samples, 36; ages overall, 19 to >244 years at the time of the growth event). As a group, the average percentage growth among the juniper trees reaches 241.6% of normal (determined against a 20-year moving average) in relative year 856. Individual juniper trees put on annual rings from 250% to 739% of normal growth. These dramatic variations are without parallel in the 6,500 years of Aegean dendrochronologies. In this geographical/climatic area, such an exceptional growth event must be due to unusually high and sustained soil moisture content and a sharp reduction in midsummer evapotranspiration³⁰; that is, for a short time there was unusually cool and wet weather. The significantly varied ages of the trees at the time of the growth event (19 years to >244 years), the specific, short-lived and highly unusual pattern of spring and summer growth, and the exceptional nature of the event in 6,500 years of Aegean chronologies, combine to suggest that it cannot be the result of a specific micro-environmental cause, such as insect attack, regional geomorphological change, or saplings from a single stand of trees forcing their way up through the forest canopy and growing without restraint. Instead, the cause must be a macro-environmental event, resulting in unusual hemisphere-wide cooler and wetter weather.

with the cutting of trees for the Midas Mound tumulus at Gordion in 718 BC. For example, the Sarıkaya Palace at Achemhöyük has wall footings of juniper and cedar that were cut (bark present) in 1752 BC, and the Waršama Palace at Kültepe was built in 1810 BC. Because documents preserved on clay in these buildings provide links with rulers from Assyria and Syria¹², the new fixed dendrochronology provides important evidence towards the resolution of a century of debate over Assyrian and Mesopotamian chronology. In particular, it renders the so-called High chronology very unlikely, and supports either a Low or lower-Middle chronology (or a new independent chronology in this range). Wood found as part of the cargo on the Kaş/Uluburun shipwreck¹³ has a last preserved ring of 1316 BC; other finds include Mycenaean pottery from Greece (the most recent material present is early Late Helladic IIIB; J. B. Rutter, personal communication), and a unique gold scarab of Nefertiti, wife of Akhenaten, pharaoh of Egypt¹⁴. These provide links to the chronologies and histories of the Aegean and Egypt, and confirm conventional 14th–12th century BC chronology^{14,15} against recent radical critiques¹⁶. Tree-ring dating now offers the route to a new, absolute, chronology of the Old World that is independent of existing assumptions, gaps in evidence, and debates.

The specific identification of the large volcanic eruption responsible for the 1628 BC climate event has implications for several recent controversies. The main candidate for the 1628 BC event has been the Minoan eruption of the volcano of Santorini/Thera in the Aegean, although other eruptions have been proposed^{17,18}. Arguments against Thera's candidacy have been based on petrological evidence suggesting that its SO₂ production was too small to account for either dramatic atmospheric cooling or the large H₂SO₄ peaks in the 17th century BC record in Greenland ice cores^{17–19}. These arguments can now be dismissed, because it is clear that petrological analysis seriously underestimates SO₂ emissions^{5,20,21}. In addition, none of the other suggested eruptions is as well dated as Thera, nor as compatible with a date in the 17th century BC²¹. The eastward distribution of volcanic products from the Thera eruption¹⁹ is consistent with a spring eruption (and thus the winter–spring date of the 1628 BC climate event). The 17th century BC growth event in the Anatolian trees was much more dramatic and clearly defined than any other growth anomaly in 6,500 years of Aegean tree-ring chronologies, including those (similar but much smaller) events that might be linked with other known large, climatically effective, volcanic events. There is likely to be a specific and local cause: the massive volcanic eruption of Thera just 840 km west of Porsuk. We suggest this as the working hypothesis; definitive confirmation must await the identification of Thera eruption products in a dated ice core, as has now been achieved for several more recent eruptions²².

If sustained, a date of 1628 BC for the Thera eruption will require a major revision of Aegean chronology at the beginning of the Late Bronze Age, raising the date of the eruption, and the associated Minoan, Mycenaean and Cycladic archaeological phases, from ~1500 BC to 1628 BC²³. Sets of material and stylistic linkages between the Aegean, Cypriot, Levantine and Egyptian cultures^{11,23,24} mean that this revision will lead to large changes in Old World chronology and history in the 18th–15th centuries BC. Longstanding assumptions and conventions in both Egyptian and Old World chronology and history will need to be re-examined. □

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Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia

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INFERTILITY resulting from a severe defect in sperm production affects 2% of men worldwide^{1,2}. Of these men with azoospermia, the absence of sperm in semen, one in eight carry *de novo* deletions for a specific region of the Y chromosome^{3–5}. A candidate gene for the Y-chromosome azoospermia factor (AZF) has been identified and named *Deleted in Azoospermia (DAZ)*⁵. Here we describe the cloning and characterization of the *Drosophila* gene *boule*, which is a homologue of *DAZ*. The two genes encode closely related proteins that contain a predicted RNA-binding motif, and both loci are expressed exclusively in the testis. Loss of *boule* function results in azoospermia; meiotic divisions are blocked, although limited spermatid differentiation occurs. Histological examination of *boule* testes with cell-cycle markers indicates that the primary defect is at the meiotic G2/M transition. These results support the hypothesis that *DAZ* is the human AZF, and indicate that *Boule* and *DAZ* have an essential meiotic function in fly and human spermatogenesis.

The *boule* (*bol*) gene was identified during screening for transposon-induced, male-sterile mutations in *Drosophila*⁶. Sequencing of several *boule* complementary DNAs indicated that *boule* encoded a protein of 228 amino acids that contained a single ribonucleoprotein (RNP)-type RNA-binding domain^{7,8} (Fig. 1a–c). Database comparisons indicated that the putative RNA-binding

domain of the *Boule* protein is most similar (42% identity) to that of the human *DAZ* protein and a closely related mouse protein, *Dazla*⁹. *Boule* also has considerable sequence similarity (33% identity) to a second region of *DAZ* and *Dazla* (Fig. 1d). This region contains a motif, termed a *DAZ* repeat, that is present once in the mouse sequence and seven times in the human protein^{5,9}. Strikingly, the positions of the RNP domain and the first *DAZ* repeat are conserved among the fly, mouse and human proteins (Fig. 1b). The human *DAZ* gene maps to a 500-kilobase region of Yq that is frequently deleted in azoospermic men; the mouse gene, like *boule*, is autosomal.

Northern analysis of RNA from adult male and female flies demonstrated that *boule* expression is limited to males (Fig. 2a). The *boule* transcript is absent from flies lacking a germ line, indicating that expression is testis specific (Fig. 2a). In *bol*¹ homozygotes this transcript, which is normally 3.0 kb in length, is truncated to just 1.1 kb (Fig. 2b, lane 6); in *bol*² flies the messenger RNA is severely reduced in abundance (Fig. 2b, lane 8). The defect in spermatogenesis seen with either allele in *trans* to a deletion is indistinguishable from that seen with *boule* homozygotes, suggesting that the phenotype of the *boule* gene could be brought about by a strong or complete loss of gene function.

To demonstrate that *boule* is active in the germ line, we performed transposon-mediated germ line transformation. The protein-coding region of *boule* was cloned into a P-element vector carrying 5' sequences from the *Drosophila* β 2-tubulin locus that direct expression in postmitotic germ cells of the testis¹⁰. When introduced into *boule* mutant flies, this construct resulted in meiotic products in more than half of the spermatid cysts. Furthermore, all spermatid cysts underwent extensive, albeit incomplete, spermiogenesis (data not shown). This partial rescue observed with the *boule* transgene suggests that the locus has a dosage-sensitive role in male germ-cell development. Indeed, minor, but reproducible, defects in spermatogenesis are seen in the testes of males bearing only one wild-type copy of *boule*.

The appearance of the wild-type *boule* transcript during the life

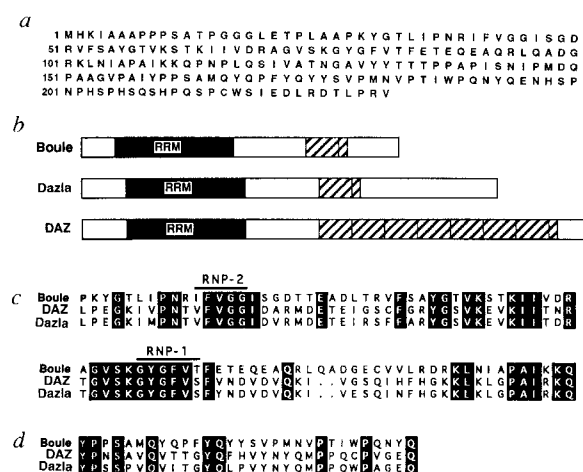
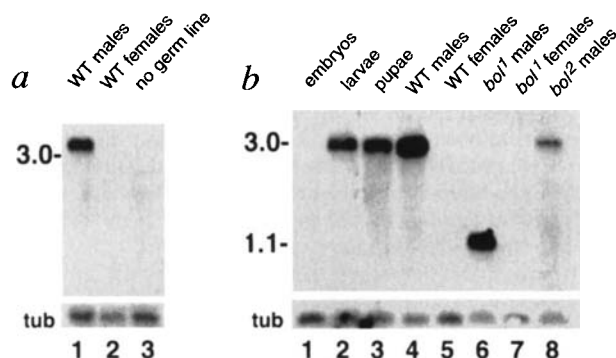


FIG. 1 Comparison of structure and sequence for Boule and DAZ proteins. *a*, Predicted Boule protein sequence. *b*, Domain organization of Boule and DAZ proteins. Scale drawings are of Boule, Dazla, (a mouse *DAZ*-like protein⁹) and the human protein *DAZ*⁵. RNA-recognition motif (RRM) RNA-binding domains and DAZ motifs are indicated by black and shaded boxes, respectively. *c*, *d*, Alignment of the RRM (*c*) and DAZ (*d*) domains of boule, DAZ and Dazla. Highlighted amino acids are identical in all three proteins. In *c*, the RNP domains are indicated by lines above the sequence. *d*, Alignment of the 24 amino-acid DAZ motifs and a 5 amino-acid extension found in Boule, DAZ (last repeat) and Dazla.

METHODS. Bacteriophage M13 sequencing was performed with an Amer-sham Sequenase kit. Sequences were assembled using AssemblyLIGN (IBI/Kodak). Homology searches used the BLASTP program²⁴.



cycle coincides with the onset of testis development and spermatogenesis¹¹ (Fig. 2b, lanes 1–4). Consistent with this pattern of expression, *boule* seems to be required for spermatogenesis only, because *boule* homozygotes have no visible defects, *boule* females are fertile, and viability in both sexes is normal. In humans, the function and expression of *DAZ* also seem to be limited to the testis.

The defects in spermatogenesis that are associated with an absence of *boule* or *DAZ* function have substantial similarities. Spermatocytes are formed in *boule* homozygotes, but fail to undergo meiotic divisions. No meiotic figures are observed, and the products of meiosis, 64-cell spermatid cysts, are absent. In individuals deleted for the *DAZ*-containing region of Yq, the postmeiotic stages of the spermatogenesis are similarly rare or absent. Some affected men display maturation arrest, in which spermatogenesis is completely, or nearly completely, stopped at the end of meiotic prophase. Others have a 'Sertoli-cell only' condition, which is defined by the lack of germ cells in testis biopsy. However, such 'Sertoli-cell only' testes frequently contain foci of germ cells in maturation arrest^{5,12}. Indeed, it has been postulated that germ-cell degeneration may be a secondary consequence of meiotic arrest in humans¹³.

The consistent phenotype of *boule* mutants, together with the accessibility of spermatogenesis in *Drosophila*^{14,15}, allowed us to define more precisely the point at which *boule* mutations disrupt the meiotic cell cycle. Close examination of mature *boule* spermatocytes indicates that they have a wild-type morphology⁶. Further-

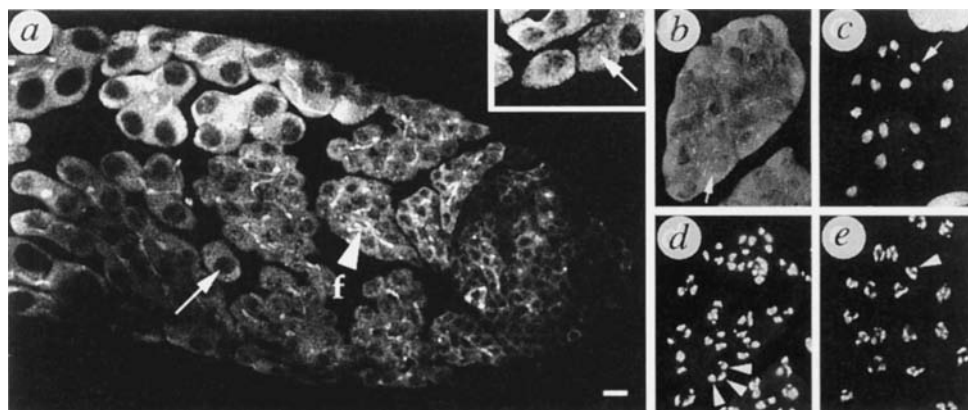
more, BrdU labelling shows that the mitotic and premeiotic S phases occur as in wild-type flies (data not shown). Comparison of the localization of cyclin A in *boule* germ cells with that in wild-type flies supports the conclusion that the meiotic prophase is normal in *boule* germ cells. In the wild type, cyclin A is exclusively cytoplasmic during the extended premeiotic G2 phase (Fig. 3a, arrow), but begins to enter nuclei at the transition between G2 and M phases (Fig. 3a inset, arrow). Cyclin A is for a short while present only in nuclei, before being degraded early in metaphase¹⁶. In *boule*, cyclin A is also initially excluded from nuclei, but then translocates into nuclei as chromosomes condense (arrow in Fig. 3b, arrowheads in Fig. 3d).

METHODS. The *boule* locus extends over more than 50 kb in polytene interval 66F on the third chromosome, and is uncovered by *Df(3L)29A6* (ref. 25). To generate the chromosomal walk for *boule*, a phage (EMBL3) genomic library (supplied by J. Tamkun) was screened with genomic probes flanking the *bol*² P-element insertion site. The *boule* cDNAs were isolated from both a testis library (Bluescript vector) (provided by T. Hazelrigg) and an adult male library (λ ZAPII vector) (provided by T. Karr). RNA isolation from flies and germ line transformation followed standard techniques²⁶. Flies lacking a germ line were generated as the progeny of females homozygous for the *oskar*³⁰¹ mutation²⁷. The P-element testis expression vector has been described previously¹⁰.

Although the meiotic prophase appears wild type in *boule* germ cells, subsequent stages are aberrant. Cyclin A, which in wild-type flies degrades rapidly after nuclear translocation, persists in *boule* nuclei (Fig. 3c). Chromosomes remain at the nuclear periphery (Fig. 3e), and centrosome separation occurs, but the centrosomes do not reach the poles and do not nucleate asters (data not shown). The nuclear lamina fails to break down in *boule* spermatocytes (Fig. 4). The simplest interpretation of these results is that the transition to metaphase does not occur in *boule* mutants. It remains possible, however, that loss of *boule* function alters the timing of events during spermatogenesis.

Spermiogenesis, which is the differentiation of spermatid structures that follows meiosis in the wild type, continues to a limited extent in *boule*. In particular, mitochondria begin to form into specialized nebenkern structures in *boule* tetraploid spermatids. A

FIG. 3 Cyclin A localization in wild-type and *boule* mutant germ cells. Fixed testis contents were stained with monoclonal cyclin A antibody (a–c) or the DNA-binding dye Hoechst (d, e); scale bar, 10 μ m. a, Wild type. The distal 10% of the testis is shown, with the tip to the right. Cyclin A is excluded from the nuclei of the growth-phase spermatocytes (arrow), and is concentrated in the branched fusome^{28,29} that interconnects the developing spermatocytes within a 16-cell cyst (f, arrowhead). The inset shows cyclin A translocation into the nuclei of mature spermatocytes (arrow) late in meiotic prophase; it becomes exclusively nuclear, and is then rapidly degraded in metaphase of meiosis¹⁴. b–e, *bol*¹ homozygotes. Paired images of cyclin A localization (b, c) and chromosome condensation (d, e). b, d, Mature *boule* spermatocytes. Cyclin A has begun to move into the nuclei of these cells (b, arrow) and the chromosomes have partly condensed (d, arrowheads). c, e, *boule* tetraploid spermatids. Cyclin A is concentrated in the nuclei of these cells (c, arrow), although the chromosomes remain at the nuclear periphery (e, arrowhead).



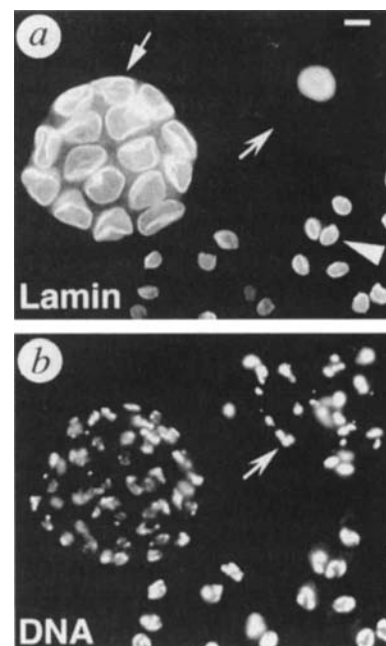
METHODS. Fluorescent studies were performed using a Zeiss Axiophot microscope. Indirect immunofluorescence of squashed testis contents from males 0–1 days old followed a previous method³⁰. Monoclonal antibodies to *Drosophila* cyclin A (from P. O'Farrell) were used undiluted. Cy3-conjugated donkey anti-mouse antiserum (Jackson Labs) was used at a 1:200 dilution.

failure to perform meiosis, accompanied by limited differentiation of tetraploid spermatids, has also been observed for mutations in two other *Drosophila* loci, namely *pelota* and *twine*^{17–20}. The biochemical function of *pelota* has not been defined, but *twine* is known to encode the meiosis-specific homologue of Cdc25 phosphatase.

The locus *twine* is an established regulator of M-phase progression. The similarity in phenotype between *boule* and *twine* suggests that *boule*, and by analogy *DAZ* in humans, controls meiotic cell divisions. As putative RNA-binding proteins, Boule and DAZ might be involved in the processing, localization or translation of mRNA^{7,8}. Although several other proteins in the RNP family have been implicated in the regulation of spermatogenesis in *Drosophila* and vertebrates, they are generally required during postmeiotic differentiation, a stage in which there is substantial translation of stored mRNAs^{21,22}.

We have shown that *boule* resembles *AZF* in loss-of-function phenotype, and *DAZ* in both testis-specific pattern of expression and amino-acid sequence. These findings provide strong support for the hypothesis that *DAZ* is *AZF*. Comparative studies of fertile and infertile men indicate that mutations in *DAZ* are extremely frequent, occurring at a frequency of at least 1 in 8,000 men, and that *DAZ* deletions contribute to cases of oligospermia, which is reduced sperm count, as well as azoospermia^{5,23}. Future investigations into the function of *DAZ* and *boule* might provide further insight into both the regulation of the meiotic cell cycle and the physiological basis of a significant determinant of human infertility. □

FIG. 4 Persistence of the nuclear lamina in *boule*. Fixed testes contents; spermatocytes and tetraploid spermatids from a *bol*¹ homozygote. The images are paired, showing staining of the same region with either: a, antibodies to nuclear lamin; or b, the DNA-binding dye Hoechst. Scale bar, 10 μ m. The nuclear lamina is clearly visible in mature *boule* spermatocytes (a, arrow). It is still intact in *boule* tetraploid spermatids (a, arrowhead) in which the mitochondria have begun to form the spermatid nebenkern. The nuclear lamina is eventually degraded, and is not present in older *boule* tetraploid spermatids (a, b notched arrows), although the lamina surrounding the large somatic cyst nucleus is still clearly visible (a, top right). **METHODS.** Sample preparation and analysis were as in Fig. 3. Monoclonal anti-lamin antibodies (from D. Glover) were used undiluted.



Functional receptor for GDNF encoded by the *c-ret* proto-oncogene

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GLIAL-CELL-LINE-DERIVED neurotrophic factor (GDNF) promotes the survival and phenotype of central dopaminergic^{1,2}, noradrenergic³ and motor neurons^{4,6}, as well as various subpopulations of peripheral sensory and sympathetic neurons^{7,8}. GDNF is structurally related to members of the transforming growth factor (TGF)- β superfamily⁹, several members of which have well-characterized receptor systems^{10,11}; however, GDNF receptors still remain undefined. Here we show that GDNF binds to, and induces tyrosine phosphorylation of, the product of the *c-ret* proto-oncogene, an orphan receptor tyrosine kinase, in a GDNF-responsive motor-neuron cell line. Ret protein could also bind GDNF and mediate survival and growth responses to GDNF upon transfection into naive fibroblasts. Moreover, high levels of *c-ret* mRNA expression were found in dopaminergic neurons of the adult substantia nigra, where exogenous GDNF protected Ret-positive neurons from 6-hydroxydopamine-induced cell death. Thus the product of the *c-ret* proto-oncogene encodes a functional receptor for GDNF that may mediate its neurotrophic effects on motor and dopaminergic neurons.

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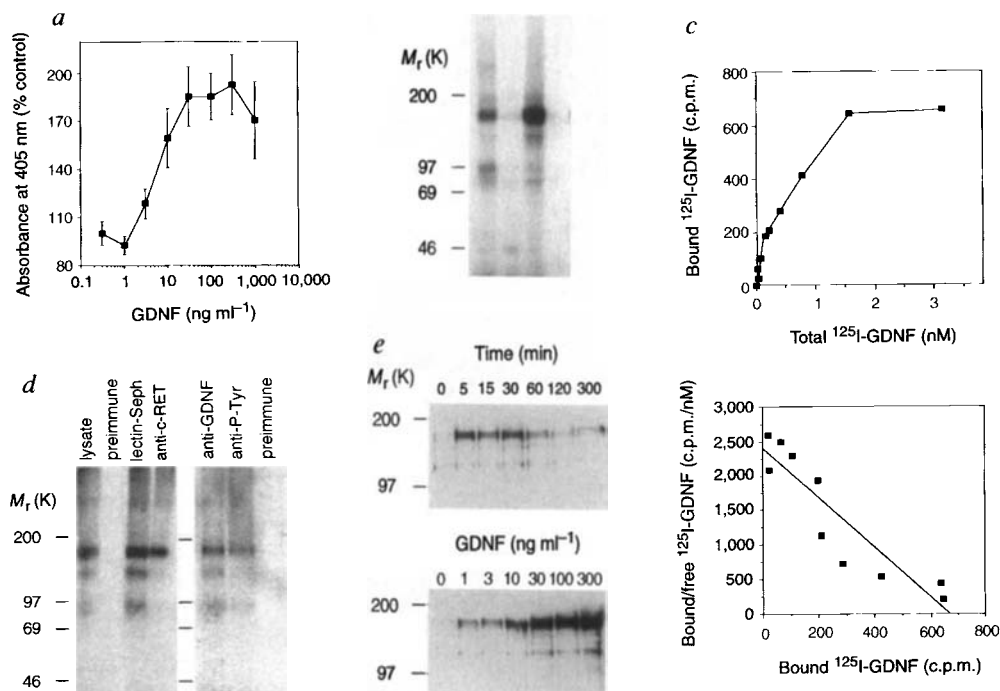
CORRESPONDENCE and requests for materials should be addressed to S.A.W. (e-mail: stevenw@pooh.su.med.edu). The *boule* cDNA sequence has been deposited in Genbank under accession number U51858.

Because GDNF promotes the survival of primary motor neurons in culture and *in vivo*, we investigated the biological responses to GDNF in a motor-neuron hybrid cell line (hereafter called MN1) derived from embryonic mouse spinal motor neurons¹². GDNF treatment of serum-deprived MN1 monolayers increased cell number in a dose-dependent manner (Fig. 1a).

FIG. 1 Ret is a functional receptor for GDNF. **a**, GDNF stimulates survival of serum-deprived MN1 cells. **b**, Cross-linking of iodinated GDNF to receptors on MN1 cells. Note that the labelling could be displaced by excess cold GDNF. **c**, Equilibrium binding assay of GDNF to the 155K receptor on MN1 cells (top) and linear transformation of the data (bottom). A K_d of $2.18 \pm 0.53 \times 10^{-10}$ M was obtained in 4 independent experiments. The results of one representative experiment are shown. **d**, Immunoprecipitation analysis of GDNF–receptor complexes in MN1 cells. GDNF-labelled binding proteins could be precipitated with lectin–Sepharose beads, or antibodies against GDNF, phosphotyrosine (P-Tyr) and Ret. Control preimmune antibodies did not immunoprecipitate GDNF receptor complexes. **e**, GDNF induces tyrosine phosphorylation of Ret in MN1 cells. Ret tyrosine phosphorylation was detected 5 min after addition of GDNF (top). Saturation of Ret tyrosine phosphorylation was observed at 30 ng ml^{-1} GDNF (bottom).

METHODS. **a**, MN1 cell monolayers were exposed to increasing concentrations of GDNF in serum-free medium and assayed 3 days later for cell survival and growth by measurement of acid phosphatase activity (Clontech). GDNF was produced and purified from baculovirus-infected insect cells as previously described⁷. **b**, GDNF was iodinated as previously described⁷ to a specific activity of 2×10^8 c.p.m. per μg . Iodinated GDNF at 10 ng ml^{-1} was allowed to bind to MN1 monolayers at 4°C for 3 h, and was subsequently crosslinked using either DSS or EDAC as crosslinking agents. Unlabelled GDNF was used at $50\times$ molar excess. Receptor complexes were fractionated by SDS–PAGE and visualized by autoradiography. **c**, Increasing concentrations of ^{125}I -GDNF were incubated with MN1 cell monolayers and, after 3 h incubation at 4°C , receptor–ligand complexes were crosslinked with EDAC at 4°C , fractionated by SDS–PAGE, and visualized by autoradiography. Gel bands corresponding to the major

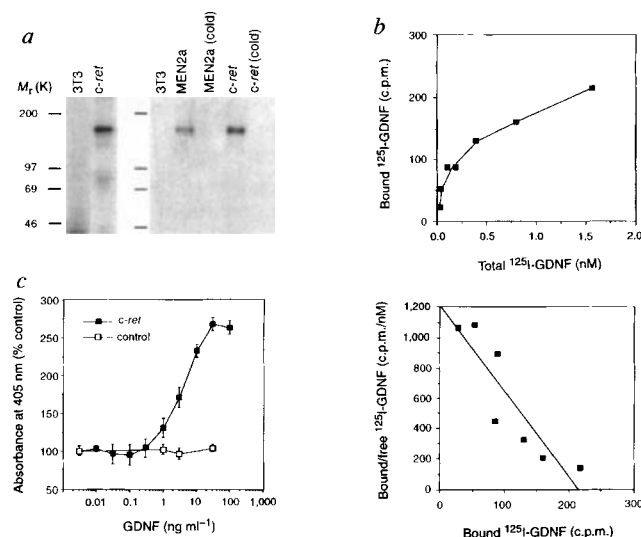
This biological response correlated with transient tyrosine phosphorylation of MAP kinases and elevated levels of *c-fos* mRNA (data not shown). The biological and biochemical responses to GDNF observed in MN1 cells indicated that these cells possess functional GDNF receptors. To identify GDNF binding components on MN1 cells, we chemically crosslinked ^{125}I -GDNF to MN1



receptor complex of 180K were excised and quantified in a γ counter. Counts of GDNF bound to receptors were plotted as a function of the total amount of labelled GDNF added (top), and a linear transformation of these data was made in a Scatchard plot (bottom). A similar analysis was also done with the 95K receptor complex (not shown). **d**, ^{125}I -GDNF was cross-linked to MN1 cells using EDAC and receptor complexes were precipitated with antibodies against GDNF⁷, lectin–Sepharose beads (Pharmacia), anti-phosphotyrosine antibodies (UBI), anti-Ret antibodies (Santa Cruz) or control antibodies from non-immune rabbits. **e**, MN1 cell monolayers were exposed to GDNF at different concentrations or for different periods of time and cell lysates were immunoprecipitated with anti-Ret antibodies and analysed by SDS–PAGE and western blotting with anti-phosphotyrosine antibodies.

FIG. 2 Ret expression confers binding and biological responsiveness to GDNF in fibroblasts. **a**, Iodinated GDNF at 10 ng ml^{-1} could be crosslinked to 3T3 cells stably transfected with a wild-type *c-ret* expression plasmid (left). Untransfected 3T3 cells (3T3) did not bind GDNF. After immunoprecipitation with anti-Ret antibodies (right), GDNF–Ret complexes could be detected in 3T3 fibroblasts transfected with MEN2a-ret and wild-type *c-ret* expression plasmids, but not in untransfected cells. The specificity of the binding was demonstrated by displacement of the labelling with $50\times$ excess unlabelled GDNF. **b**, Equilibrium binding assay of GDNF to Ret ectopically expressed on 3T3 fibroblasts (top) and Scatchard transformation of the data (bottom). A K_d of $2.75 \pm 1.16 \times 10^{-10}$ M was obtained in 3 independent experiments. The results of one representative experiment are shown. **c**, GDNF promotes survival and growth responses in 3T3 fibroblasts stably transfected with a *c-ret* expression plasmid. Untransfected cells did not respond to GDNF.

METHODS. For *c-ret* expression in transfected cells, human wild-type *c-ret* and MEN2a-ret cDNAs were subcloned in pcDNA3 (Invitrogen). The MEN2a-ret allele corresponded to the point mutation C634R. Cold GDNF was used at $50\times$ molar excess. Saturation binding assays to Ret expressed on fibroblasts were performed as described in the Fig. 1 legend. For survival and growth assays, cells were cultured for 6 days in serum-free medium supplemented with the indicated concentrations of GDNF; medium and GDNF were replaced every 2 days. Cell survival and growth was quantified by measurement of acid phosphatase activity (Clontech).



monolayers and visualized the resulting complexes by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A major complex of relative molecular mass 180,000 (M_r 180K) was crosslinked by ethyl-dimethylaminopropyl carbodiimide (EDAC) which approximately corresponds to a binding protein of 155K cross-linked to a glycosylated GDNF monomer (M_r ~25K) (Fig. 1b). An

excess of cold ligand displaced 125 I-GDNF from this receptor complex, indicating that the labelling represented specific binding. The use of another crosslinker, disuccinimidyl suberate (DSS), also resulted in the labelling of this component, and of a minor component of 95K, corresponding to a binding protein of approximately 70K (Fig. 1b). The binding affinity of GDNF for its

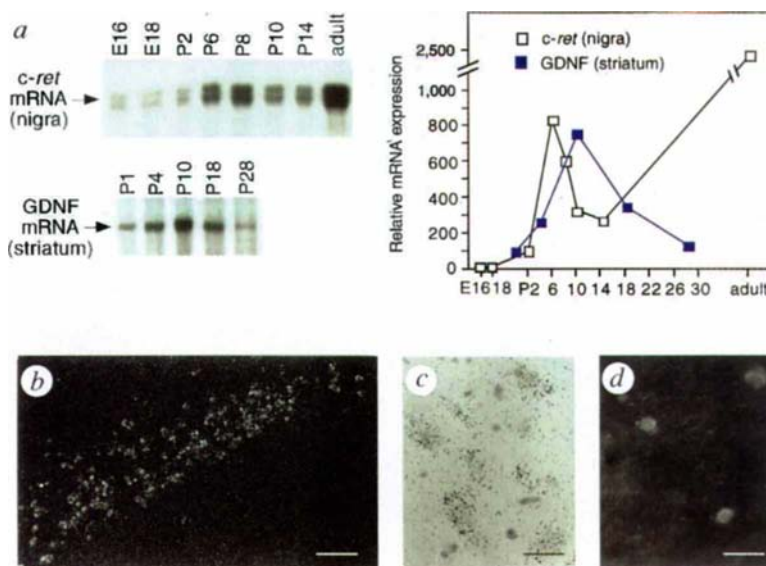


FIG. 3 Expression of *c-ret* mRNA in adult and developing substantia nigra. **a**, RNase protection assay (RPA) of *c-ret* mRNA expression during the development of the rat ventral mesencephalon (nigra), and of *GDNF* mRNA expression in the developing striatum. In the plot to the right, mRNA expression is indicated in arbitrary units where 100 corresponds to the level of expression in the respective regions in newborn animals. **b**, Dark-field autoradiogram of *c-ret* mRNA expression in the adult substantia nigra analysed by *in situ* hybridization. Scale bar, 40 μ m. **c**, Bright-field autoradiogram showing substantia nigra neurons containing *c-ret* mRNA. Scale bar, 7.5 μ m. **d**, Immunohistochemical analysis of Ret protein expression in the adult substantia nigra. Scale bar, 27 μ m. **METHODS**. A rat *c-ret* riboprobe was generated using as template a cDNA

fragment obtained by PCR with primers based on sequences U22513 and U22514 (Genbank accession numbers), resulting in a 262-bp fragment from the extracellular domain of rat *c-ret*. For mRNA quantification, a glyceraldehyde-3-P dehydrogenase (GAPDH) riboprobe was included in the RPA, and values of relative mRNA expression, obtained after densitometric scanning of gel autoradiograms, were normalized using the GAPDH signal of each RNA sample. RPA for *GDNF* mRNA has been previously described⁷. *In situ* hybridization and immunohistochemistry were performed as previously described^{23,24}. Ret protein was detected using a hamster monoclonal anti-mouse Ret antibody that also recognizes rat Ret¹⁷, followed by fluorescein-conjugated rabbit anti-hamster secondary antibodies (Southern Biotechnologies).

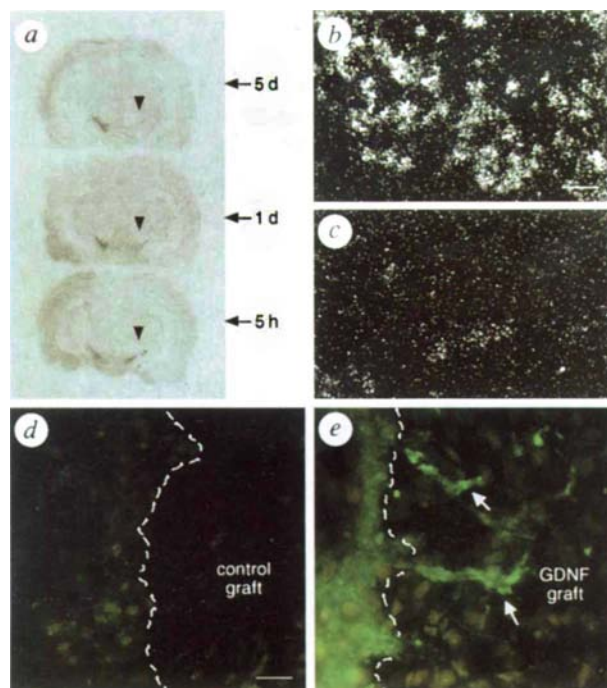


FIG. 4 Ret is expressed in GDNF-responsive substantia nigra dopaminergic neurons. **a**, Autoradiogram showing *in situ* hybridization for *c-ret* mRNA in the adult rat brain after a unilateral lesion with 6-OHDA. The injection of this toxic dopamine analogue in the medial forebrain bundle ensures that only cells which actively take up and retrogradely transport dopamine will be compromised. Note the reduction of the labelling for *c-ret* mRNA in the lesioned substantia nigra (arrowhead) 1 day and 5 days following the lesion. Dark-field autoradiograms (**b** and **c**) show *c-ret* mRNA expression at higher magnification in control (**b**) and lesioned (**c**) substantia nigra 5 days after unilateral injection of 6-OHDA. Scale bar, 25 μ m. **d**, Immunohistochemical analysis of Ret protein expression in the adult substantia nigra after lesion with 6-OHDA and grafting of mock-transfected fibroblasts (control graft). The border of the transplant is outlined. Note the nearly complete absence of Ret-LI caused by the lesion. Scale bar, 20 μ m. **e**, Grafting of GDNF-expressing fibroblast cells rescues Ret-LI. Note Ret-positive fibres surrounding and entering the GDNF-producing graft (arrows). Magnification is as in **d**. **METHODS**. Lesions of dopaminergic neurons of the substantia nigra were performed by stereotaxic injections of 8 μ g 6-OHDA in the medial forebrain bundle at the following coordinates: 1.6 mm caudal to bregma; 1.3 mm lateral to midline; and 8.4 mm under the dural surface with the incisor bar 5 mm over the interaural line. Animals were pretreated with 25 mg per kg desipramine (intraperitoneal) 30 min before 6-OHDA injection. GDNF-expressing fibroblast cells (0.75×10^6) in 3 μ l of medium were injected supranigrally at the following coordinates: 3.1 mm from interaural line; 2 mm lateral to midline; and 7 mm under the dural surface, with the incisor bar at -3.3 mm. The generation and characterization of GDNF-expressing fibroblasts have been described previously³.

receptors in MN1 cells was assessed in a dose–response equilibrium binding assay (Fig. 1c). GDNF binding to either receptor component was saturable, and a linear transformation of these data indicated a dissociation constant. (K_d) of $2.18 \pm 0.53 \times 10^{-10}$ M (4 experiments) for the major 155 K receptor component (Fig. 1c), and of $6.67 \pm 3.01 \times 10^{-10}$ M (2 experiments) for the 70K component (data not shown). GDNF receptor complexes could be recovered by immunoprecipitation with anti-GDNF antibodies or by binding to lectin–Sepharose beads (Fig. 1d). Unexpectedly, the 180K receptor complex could also be recovered by immunoprecipitation with anti-phosphotyrosine antibodies (Fig. 1d), indicating that the GDNF binding protein in this complex could be a receptor tyrosine kinase.

The product of the *c-ret* proto-oncogene is a receptor tyrosine kinase that is highly expressed in primary motor neurons^{13,14} and is of similar size to the major GDNF receptor component detected in MN1 cells¹⁵. Therefore, we tested whether the 180K band represented a Ret–GDNF crosslinked complex by immunoprecipitation with anti-Ret antibodies. An antipeptide Ret rabbit antiserum readily immunoprecipitated the major 180K ligand–receptor complex in MN1 cells (Fig. 1d), but several unrelated monoclonal and polyclonal antibodies used as controls failed to bring down this complex (Fig. 1d, and data not shown). We investigated whether GDNF could stimulate activation of the Ret receptor by examining tyrosine phosphorylation of the Ret protein in MN1 cells. GDNF treatment stimulated rapid Ret tyrosine phosphorylation in MN1 cells (Fig. 1e). Maximal phosphorylation was reached 5 min after GDNF treatment, and lasted for at least 60 min. A dose–response analysis of GDNF-induced Ret phosphorylation in MN1 cells showed maximal phosphorylation at 30 ng ml^{-1} GDNF (Fig. 1e), which is similar to the response of both serum-deprived MN1 cells (Fig. 1a) and embryonic sympathetic neurons⁷ to GDNF.

We next examined whether expression of the *c-ret* gene product could allow binding of GDNF to cells unresponsive to the factor. To this end, GDNF binding and crosslinking experiments were performed with naive 3T3 fibroblasts, and 3T3 cells stably transfected with either a wild-type *c-ret* or an oncogenic form of this gene found in MEN2a patients¹⁶. Untransfected 3T3 fibroblasts did not bind GDNF at the concentrations tested, although, after transfection with a *c-ret* expression plasmid, iodinated GDNF strongly labelled a receptor component of 155K (Fig. 2a). After immunoprecipitation with Ret antibodies, GDNF–Ret complexes could be detected in both MEN2a-*ret* and *c-ret* transfected 3T3 fibroblasts, but not in untransfected cells (Fig. 2a). The labelling could be displaced by excess cold GDNF, indicating that it represented specific GDNF binding (Fig. 2a). The K_d of GDNF binding to Ret in transfected fibroblasts, as determined by equilibrium binding assay and Scatchard transformation, was $2.75 \pm 1.16 \times 10^{-10}$ M (3 experiments) (Fig. 2b), and was similar to that obtained in MN1 cells. In addition, very low levels of the 95K GDNF–receptor complex similar to that seen in MN1 cells could also be recovered. The fact that this component was affinity labelled by GDNF only after *c-ret* transfection suggests an interaction between these two receptor components. We also investigated whether *c-ret* could mediate a biological response to GDNF on transfection in non-responsive cells. Survival and growth responses to GDNF were investigated in untransfected and *c-ret* transfected 3T3 fibroblasts cultured in serum-free medium. GDNF elicited a dose-dependent increase in cell number in *c-ret* transfected, but not in untransfected, 3T3 cells (Fig. 2c), which was comparable to that previously observed in serum-deprived MN1 cells. Thus, although our data suggest the presence of additional GDNF-binding proteins, GDNF activity in 3T3 cells was seen only after *c-ret* transfection, indicating that *c-ret* expression imparts biological responsiveness to GDNF.

Having demonstrated that *c-ret* expression confers GDNF responsiveness, we investigated whether the *c-ret* product can mediate the neurotrophic effects of GDNF in the brain. We examined the expression of *c-ret* in different regions of the rat

central nervous system. High *c-ret* mRNA expression was found in the adult rat spinal cord, pons, medulla, hypothalamus, thalamus and cerebellum (data not shown), but there were barely detectable levels in striatum, hippocampus and cerebral cortex (data not shown). In the ventral mesencephalon, which contains the cell bodies of GDNF-responsive dopaminergic neurons, *c-ret* mRNA levels increased progressively during postnatal development (Fig. 3a). A peak of expression was detected between postnatal day 6 (P6) and P8, at which time axons of dopaminergic neurons of the substantia nigra begin functional innervation of the striatum, and coincident with an increase in GDNF mRNA expression in this target region (Fig. 3a). *In situ* hybridization on sections through the adult substantia nigra revealed strong labelling over neurons throughout this structure (Fig. 3b, c). In addition, cells positive for Ret-like immunoreactivity (Ret-LI) were found throughout the adult substantia nigra, with strong labelling over cell bodies (Fig. 3d). To establish that *c-ret* expression in the adult substantia nigra was confined to dopaminergic neurons, we selectively lesioned these cells with a unilateral injection of 6-hydroxydopamine (6-OHDA), and analysed *c-ret* mRNA expression by *in situ* hybridization. No difference could be seen between ipsi- and contralateral sides in *c-ret* mRNA expression 5 h after the lesion (Fig. 4a). However, *c-ret* mRNA expression was markedly reduced in the lesioned substantia nigra one day after 6-OHDA treatment, and nearly absent five days after the lesion (Fig. 4a–c); *c-ret* mRNA expression in the side contralateral to the lesion was not affected. This indicated that, in the adult substantia nigra, *c-ret* mRNA expression was confined to dopaminergic neurons.

Finally, we investigated whether Ret-expressing neurons of the adult substantia nigra responded to GDNF. For this, we performed 6-OHDA lesions of nigral dopaminergic neurons, and examined whether grafts of GDNF-expressing fibroblasts induced responses on Ret immunoreactive cells. In lesioned rats that received a graft of control fibroblasts, no Ret-LI could be detected, indicating a depletion of Ret-expressing cells by selective lesion of dopaminergic neurons in the adult substantia nigra (Fig. 4d). However, Ret-LI could be rescued by the GDNF-expressing graft, where Ret immunopositive fibres could be seen surrounding and penetrating the transplant (Fig. 4e). The rescue of Ret-LI-positive cells and sprouting in the animals grafted with GDNF-expressing fibroblasts was similar to that of tyrosine hydroxylase immunoreactivity (data not shown), demonstrating that Ret-expressing adult dopaminergic neurons respond to GDNF.

Our results indicate that the Ret receptor tyrosine kinase is a signal-transducing receptor for GDNF. This finding is surprising, given that all receptors for members of the TGF- β superfamily characterized thus far are receptor serine-threonine kinases^{10,11}. The ability of GDNF to interact with a receptor tyrosine kinase indicates a further functional divergence from other members of the TGF- β superfamily. Our results do not rule out the possibility that some of the activities of GDNF may be mediated by receptors with intrinsic serine-threonine kinase activity, however. Conversely, these findings could suggest that other TGF- β superfamily members may also use receptor tyrosine kinases. The expression of *c-ret* in cranial ganglia, autonomic and sensory neurons, and in several regions of the central nervous system, has led to the hypothesis that Ret is a receptor for a neurotrophic factor required for differentiation or survival of certain lineages of the mammalian nervous system¹³. Based on the evidence presented here, we propose that GDNF could be one such factor. Ret is one of the earliest markers expressed by postmigratory neural crest cells¹⁷. In line with this, several subpopulations of neurons derived from the neural crest have been found to respond to GDNF, including sympathetic neurons and a subpopulation of sensory neurons in the dorsal root ganglia^{7,8}. Because Ret is highly expressed in the enteric nervous system¹³, and *c-ret*^{-/-} mice lack enteric neurons¹⁸, our data predict that GDNF, which is highly expressed in the gastrointestinal tract^{7,19}, could also be a crucial

signal for the development of enteric neurons. Germline mutations of *c-ret* have been found to contribute to developmental abnormalities in a fraction of cases of Hirschsprung's disease, a congenital intestinal aganglionosis^{20,21}. Because additional loci for Hirschsprung's disease other than *c-ret* have been proposed²², our results prompt examination of the possibility that some of these loci might correspond to the *GDNF* gene. Finally, our findings indicate that Ret may mediate the neurotrophic effects of GDNF in the central nervous system. The expression of *c-ret* in motor and dopaminergic neurons is in accordance with the biological activities of GDNF on these cells. In particular, the patterns of mRNA expression in the developing brain presented here support the notion that interactions between GDNF and Ret play a trophic role in the innervation of striatum by dopaminergic substantia nigra neurons. Moreover, the ability of GDNF to protect Ret-positive neurons of the substantia nigra from 6-OHDA-induced cell death suggests that Ret mediates survival responses in these cells. Interestingly, however, the high levels of *c-ret* mRNA expression found in the adult substantia nigra, at a time when GDNF is minimally expressed in the brain³, suggest that cognate ligands for Ret other than GDNF may be present in the adult brain. The neuroprotective activities of GDNF have stimulated great interest in this factor as a candidate therapeutic agent for the treatment of Parkinson's disease. New approaches for the treatment of this and other neurodegenerative diseases may be developed by targeting Ret or the signalling pathway that is initiated when this receptor is activated. □

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GDNF signalling through the Ret receptor tyrosine kinase

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MUTATIONAL analysis in humans and mice has demonstrated that Ret, the product of the *c-ret* proto-oncogene, a member of the receptor tyrosine kinase (RTK) superfamily¹, is essential for development of the enteric nervous system and kidney^{2–6}. Despite the established role of Ret in mammalian embryogenesis, its cognate ligand(s) is currently unknown. Here we demonstrate, by using a *Xenopus* embryo bioassay, that glial-cell-line-derived neurotrophic factor (GDNF)⁷, a distant member of the transforming growth factor(TGF)- β superfamily, signals through the Ret RTK. Furthermore, using explant cultures from wild-type and Ret-deficient mouse embryos⁴, we show that normal *c-ret* function is necessary for GDNF signalling in the peripheral nervous system. Our data strongly suggest that Ret is a functional receptor for GDNF, and that GDNF, in addition to its potential role in the differentiation and survival of central nervous system neurons^{8–12}, has profound effects on kidney organogenesis and the development of the peripheral nervous system.

Activation of mitogen-activated protein kinase (MAPK) in animal cap cells isolated from blastula-stage *Xenopus* embryos, which normally give rise to epidermal tissue, is necessary and sufficient to induce mesoderm formation^{13–15}. Because RTKs are potent activators of MAPK¹⁶, we considered that signalling through the Ret RTK would also lead to mesoderm formation in animal caps. To test this, *in vitro* synthesized RNA that encodes a constitutively active form of the Ret receptor (Ret^{C634R}, in which a cysteine residue is replaced by an arginine at position 634 in the extracellular domain)^{17,18} was injected into one-cell stage *Xenopus* embryos. Mesoderm formation was then assayed in animal caps dissected from blastula-stage embryos. Animal caps from control (uninjected) embryos, or embryos expressing wild-type Ret, differentiated into atypical epidermis as shown by their spherical shape, lack of mesodermal tissues, and failure to express Xbra (Fig. 1a–d, g). However, most (~82%) of the caps expressing Ret^{C634R} underwent elongation movements (Fig. 1e) similar to those caused by the addition of basic fibroblast growth factor, which is known to induce mesoderm formation. By the tadpole stage, histological analysis showed that these caps contained various types of mesodermal tissues, including mesenchyme, muscle and pronephros (Fig. 1f). The formation of mesoderm in caps expressing Ret^{C634R} was confirmed by the induction of Xbra RNA, a widespread mesodermal marker of early gastrula *Xenopus* embryos (Fig. 1g)¹⁹. These findings indicate that activation of the Ret signal transduction pathway is sufficient to induce formation of mesoderm in animal cap explants, and led us to postulate that coexpression of the wild-type Ret receptor and its putative ligand(s) would also lead to mesoderm formation.

GDNF is a distant member of the TGF- β superfamily, with potent neurotrophic effects on dopaminergic and motor neurons^{7,10–12}. Although the *in vivo* function of GDNF is at present unclear, recent expression studies^{20,21} have suggested a role in mammalian organogenesis, in particular the development of the kidney and gut, the two embryonic regions expressing the highest levels of GDNF mRNA. Because the *ret.k*[–] mutation in mice leads to characteristic defects in the development of the nervous system of the gut and kidney⁴, we postulate that Ret might be a functional receptor for GDNF. To test this hypothesis, we assayed the competence of these molecules to induce mesoderm formation

when coexpressed in *Xenopus* animal cap explants. RNAs encoding murine Ret and human GDNF were injected, separately or in combination, into one-cell-stage *Xenopus* embryos, and mesoderm formation was assayed in animal caps dissected from blastula-stage embryos. Only a small proportion of animal caps expressing either Ret or GDNF alone were induced to form mesoderm (2.7% and 1.3%, respectively; Fig. 2b–e, k). However, coexpression of both molecules led to a high percentage of animal caps (53%) containing mesodermal tissues, as assessed by the morphological and histological criteria described above (Fig. 2f, g, k). We also demonstrated that coinjection of Ret and GDNF RNAs also led to Xbra expression, whereas injection of either Ret or GDNF RNA alone failed to do so (Fig. 2j). Qualitatively similar results were obtained from animal caps expressing Ret and exposed to recombinant human GDNF (50 ng ml⁻¹; data not shown), suggesting that GDNF acts in a non-cell-autonomous manner. Finally, the specificity of interaction between Ret and GDNF was established by experiments in which co-injection of RNAs encoding GDNF and other members of the RTK superfamily, such as Sek-1 (ref. 22), failed to induce mesoderm formation in the derivative animal caps (Fig. 2h, i, k). These data demonstrate that GDNF is capable of specific activation of the Ret signal transduction pathway, and suggest that it is a functional ligand for the Ret RTK.

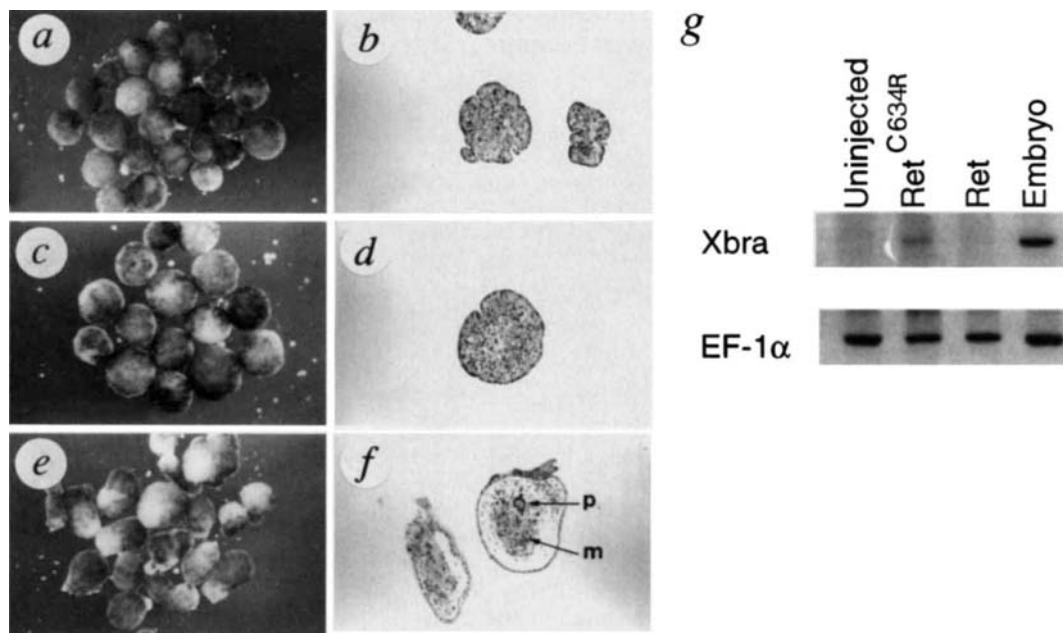
To investigate the functional interaction between GDNF and Ret, *Xenopus* oocytes expressing the murine Ret RTK were briefly treated with recombinant human GDNF, and Ret-specific tyrosine phosphorylation was assayed by immunoprecipitation and western blotting. Addition of GDNF led to a significant increase of tyrosine phosphorylation of the mature form of Ret compared to non-treated Ret-expressing oocytes (Fig. 3a). Thus Ret can function as a receptor for GDNF. To test this directly, we examined the specific binding of ¹²⁵I-labelled GDNF to oocytes expressing Ret. Incubation of oocytes expressing Ret with two different concentrations of ¹²⁵I-labelled GDNF resulted in specific

binding compared to control (water-injected) oocytes (Fig. 3b). Overall, our data demonstrate specific and direct interaction between GDNF and Ret.

Based on the phenotypic analysis of Ret-deficient mouse embryos, we have suggested that *c-ret* encodes a receptor for a factor produced by the metanephric mesenchyme, which mediates the inductive effects of this tissue on the ureteric bud and its branches^{4,6,23}. To examine further whether GDNF fulfils the criteria for a functional Ret ligand *in vivo*, we analysed the relative distribution of Ret and GDNF mRNAs in serial sections of mouse metanephroi at various developmental stages. Our data show that *c-ret* is clearly expressed by the epithelial cells of the ureteric bud, whereas GDNF mRNA is localized in the neighbouring cells of the metanephric mesenchyme (Fig. 4a, b).

A testable prediction of the functional interaction between Ret and GDNF is that cells from *ret.k⁻* homozygous embryos are unable to respond to GDNF. Explants derived from the nephrogenic region of embryonic day (E) 11.0–11.5 wild-type mouse embryos contain mainly two types of cells that express high levels of *c-ret* mRNA: the epithelial cells of the ureteric bud and its branches, and a population of autonomic neuroblasts^{24,25}. Agarose beads soaked in GDNF had a dramatic effect on axonal outgrowth by neuroblasts present in the explants, as a large number of axons were reproducibly observed growing towards and eventually encapsulating the GDNF beads (Fig. 4c). This observation is consistent with previous reports demonstrating that GDNF has a neurotrophic effect on autonomic neuroblasts *in vitro*²⁶. However, such a response was not observed in explants from *ret.k⁻* homozygous embryos (Fig. 4d), despite the presence of the normal complement of sympathetic neuroblasts at the time of dissection⁵ (P.D. and V.P., unpublished observations). This suggests that the lack of neuronal response in mutant explants is exclusively due to the absence of the Ret RTK. A dramatic difference in the branching of the ureteric bud was also observed between wild-type and Ret-deficient explants in the presence of

FIG. 1 Induction of mesoderm in blastula animal caps by activated Ret (Ret^{C634R}). Morphological (a, c, e), histological (b, d, f) and molecular (g) analysis of mesoderm formation in animal caps derived from uninjected embryos (a, b), and embryos injected with 2 ng wild-type human Ret RNA (c, d) or Ret^{C634R} RNA (e, f). Animal caps derived from embryos injected with Ret^{C634R} RNA undergo elongation and generate mesenchyme, muscle and pronephros. No elongation or mesodermal tissues were observed in animal caps derived from uninjected embryos or embryos injected with wild-type Ret RNA. g, Reverse transcription with polymerase chain reaction (RT-PCR) analysis of Xbra expression in animal caps. Injection of Ret^{C634R} RNA causes activation of Xbra



expression. No Xbra RNA was detected in animal caps from uninjected or wild-type Ret RNA-injected embryos. EF-1α was used as an RNA extraction and reverse transcription control. Labels: m, muscle; p, pronephros. METHODS. *Xenopus* embryos were obtained by standard techniques and staged as described.³⁰ Complementary DNA encoding human wild-type Ret and Ret^{C634R}, which is identical to wild-type Ret except that cysteine 634 of the extracellular domain has been replaced by an arginine, were subcloned into pSP64T vector linearized with XbaI. RNA was synthesized *in vitro* by SP6

polymerase according to the manufacturer's specifications. Embryos were injected at the one-cell stage by standard protocols, and animal caps were dissected at stage 8 using fine watchmaker's forceps, and cultured in 75% NAM. Analysis of morphogenetic movements was done at the equivalent of stage 16, whereas histological analysis was performed on animal caps at the equivalent of stage 40. Thirty animal caps were frozen at stage 11 for RT-PCR analysis²⁷, and PCR products were analysed by 2% agarose gel electrophoresis.

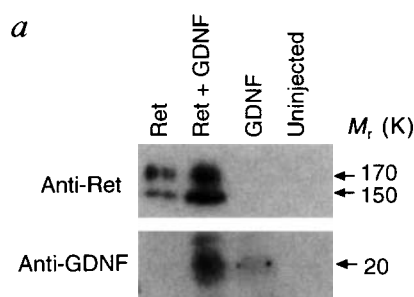
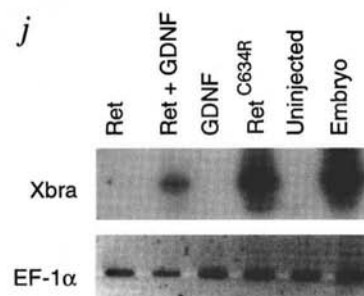
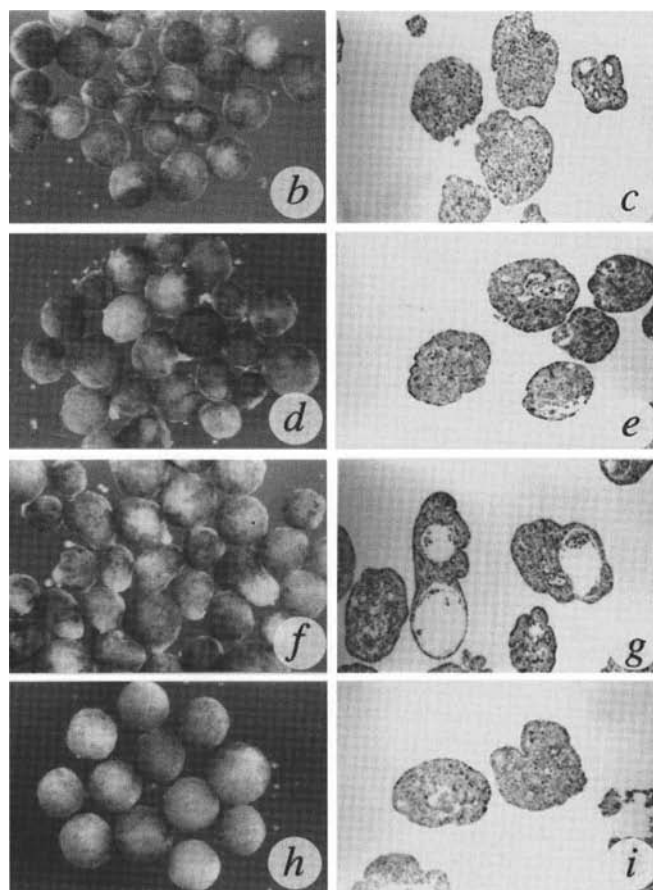


FIG. 2 Mesoderm induction in animal caps by coexpression of wild-type Ret and GDNF. **a**, Mature forms of Ret and GDNF are produced in *Xenopus* oocytes injected with *in vitro* synthesized RNA. Western blot analysis of lysates from oocytes injected with Ret RNA using Ret-specific polyclonal antibodies identified two proteins of M_r 150K and 170K. These sizes are in good agreement with a partially and a fully glycosylated form of the Ret receptor expressed on the plasma membranes of mouse fibroblasts²⁸. Similar analysis indicated that *Xenopus* oocytes injected with GDNF RNA express a protein of M_r 20K, in good agreement with the predicted size of mature GDNF⁷. **b–j**, Coexpression of Ret and GDNF in blastula animal caps results in mesoderm formation. Morphological (**b**, **d**, **f**, **h**), histological (**c**, **e**, **g**, **i**), and molecular (**j**) analysis of mesoderm formation in animal caps derived from embryos injected with 2 ng of Ret RNA (**b**, **c**), GDNF RNA (**d**, **e**), Ret and GDNF RNAs (**f**, **g**), and Sek-1 and GDNF RNAs (**h**, **i**). Only animal caps derived from embryos coinjected with Ret and GDNF RNAs underwent elongation and generated mesodermal tissues. **j**, RT-PCR analysis of Xbra expression in animal caps. Coinjection of Ret and GDNF RNAs leads to activation of Xbra expression. No Xbra expression was detected in animal caps derived from embryos injected with Ret or GDNF RNA alone (**j**), or coinjected with Sek-1 and GDNF RNAs (not shown). **k**, Animal caps induced after RNA injection at the one-cell-stage embryo. Numbers of animal caps analysed for elongation or histological evidence of mesoderm formation are given in brackets, and results are based on three independent experiments. **METHODS**. *Xenopus* oocytes were injected with 2 ng of *in vitro* synthesized RNA. Western blot analysis was performed using anti-Ret-specific and anti-GDNF-specific antibodies (Santa Cruz). One-cell-stage *Xenopus* embryo injections and subsequent analysis were performed as described in Fig. 1, except that Xbra expression was analysed by Southern blotting of PCR products.



k

RNA injected	Animal caps induced (%)
Ret	2.7 (2/78)
GDNF	1.3 (1/78)
Ret + GDNF	53 (55/103)
Sek-1	0 (0/63)
Sek-1 + GDNF	0 (0/59)
Ret ^{C634R}	82 (33/40)
Uninjected	0 (0/72)

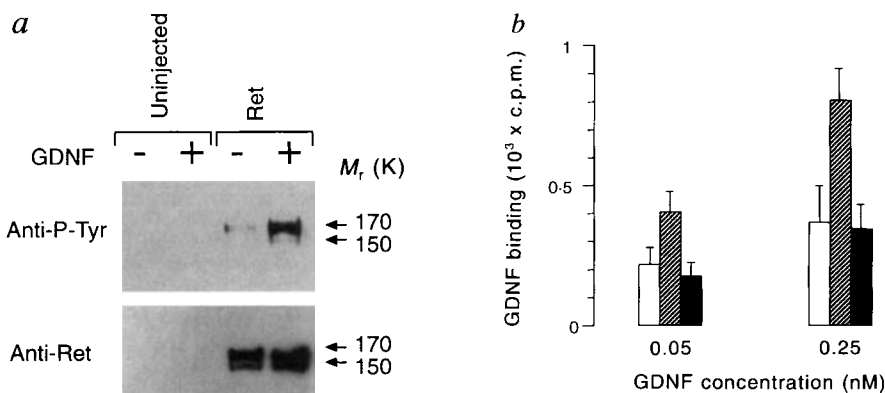
GDNF. However, because the ureteric bud is already smaller at the time of dissection, it is not clear whether the observed difference in the branching phenotype is exclusively due to the lack of GDNF response (K. Sainio and H.S., manuscript in preparation). Overall, our explant analysis shows that the presence of a functional Ret receptor is required for GDNF signalling *in vivo*. This, in conjunction with the *Xenopus* embryo bioassay and our phosphorylation and binding studies, indicates that Ret is necessary and sufficient for GDNF signalling. This strongly suggests that the *c-ret* locus encodes a functional receptor for GDNF.

GDNF was originally identified as a potent neurotrophic factor for neurons of the central nervous system (CNS). However, the identification of Ret as a functional receptor for GDNF, combined with the phenotypic effects of the *ret.k⁻* mutation, strongly suggests that GDNF is essential for kidney morphogenesis and the development of the peripheral nervous system (PNS). Consistent with this hypothesis, we have recently observed that GDNF has dramatic effects on the survival and differentiation of enteric neuroblasts purified from the bowel of mouse embryos and cultured *in vitro* (M.G. and V.P., unpublished data). All cell

types known to respond to GDNF *in vivo* and *in vitro* (motor, dopaminergic and adrenergic neurons of the CNS; autonomic, enteric and subsets of sensory neuroblasts and neurons of the PNS; and the epithelial cells of the ureteric bud and its branches) express high levels of *c-ret*. It is therefore likely that signalling by GDNF in these cell types is mediated by the Ret RTK. Because

FIG. 3 Tyrosine phosphorylation of Ret and specific binding of GDNF. **a**, GDNF-induced tyrosine phosphorylation of Ret expressed in *Xenopus* oocytes. **b**, Binding of 125 I-labelled GDNF to oocytes injected with water (white bars) or Ret RNA with (black bars) or without (hatched bars) 5 nM unlabelled GDNF.

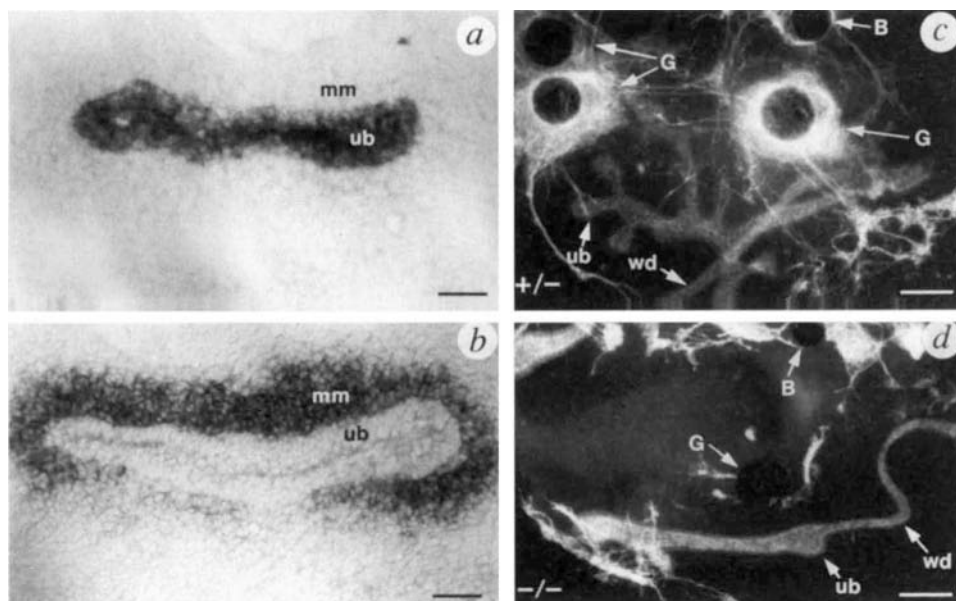
METHODS. For the phosphorylation experiments, whole-cell lysates were prepared from 50 oocytes uninjected or injected 40 h previously with 20 ng murine Ret RNA and incubated with human GDNF (50 ng ml $^{-1}$; PeproTech EC) for 5 min. These lysates were immunoprecipitated with anti-Ret antibodies (C19; Santa Cruz) and the immunoprecipitation products were subsequently analysed by western blotting using an anti-phosphotyrosine (anti-P-Tyr) monoclonal antibody (PY54; Affinity). As a loading control the membrane was stripped and reprobed with anti-Ret polyclonal antibodies. For the binding experiments, groups of 3–5 oocytes injected with water (control) or 20 ng murine Ret RNA were incubated with two different



concentrations of 125 I-labelled human GDNF (at 0°C for 3 h in the presence of bovine serum albumin, 1 mg ml $^{-1}$) 48 h after injection. Human GDNF (5 nM) was used as a specific competitor.

FIG. 4 Ret is necessary for GDNF signalling. During kidney morphogenesis, *c-ret* and GDNF are expressed by abutting cellular populations. In E11.5–12.0 mouse embryos, Ret mRNA is localized in the epithelial cells of the branching ureteric bud (ub) (**a**), whereas GDNF is produced by the surrounding cells of the undifferentiated metanephric mesenchyme (mm) (**b**). This pattern of expression is maintained throughout kidney organogenesis (not shown). **c**, **d**, Effects of GDNF on nephrogenic explants from heterozygous (**c**) or homozygous *ret.k $^{-}$* mutant (**d**) embryos. In explants from heterozygous embryos, GDNF-soaked beads (G) promote an axonal outgrowth response (halo of fluorescent labelling around the beads) from the resident autonomic neuroblasts. Significantly weaker response was observed around BSA-soaked beads (B). No axonal response to GDNF- or BSA-soaked beads was detected in mutant explants. There is a great difference in the branching of the ureteric bud between heterozygous and mutant explants; wd, wolffian duct. Scale bars: **a** and **b**, 87 μ m; **c** and **d**, 348 μ m.

METHODS. A murine GDNF cDNA was isolated from an E13.5 mouse embryo brain library using a rat GDNF cDNA (C.V.M.-G. and V.P., unpublished data) as a probe. *In situ* hybridization histochemistry with digoxigenin-labelled probes was performed on serial sections of the nephrogenic region of E11.5 mouse embryos according to standard protocols. Nephrogenic regions (including the gonadal ridge, mesonephros, wolffian duct and the



metanephric mesenchyme) were dissected from E11.0–11.5 embryos of heterozygous *ret.k $^{-}$* parents and were cultured organotypically in serum-free medium (F12). Colour-coded agarose beads (Affi-gel Blue Gel 100–200 mesh; BioRad) soaked in recombinant human GDNF (50 ng ml $^{-1}$; Pepro Tech EC) or BSA were placed in the nephrogenic explants. Explants were cultured for 48 h and processed for whole-mount immunofluorescence using anti-L1 antibodies²⁹. Explants from all *ret.k $^{-}$* mutant embryos identified in four litters of heterozygous parents failed to show a response to GDNF.

other members of the TGF- β family of growth factors signal through serine–threonine kinase receptors, it is intriguing that GDNF signals through an RTK. We believe that this emphasizes further the distant relationship of GDNF to other members of this family of growth factors, and raises the possibility that additional molecules similar to TGF- β could bind to and activate RTKs. However, we cannot exclude the possibility that GDNF also binds to and signals through additional serine–threonine kinase receptors. □

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Synaptic strengthening through activation of Ca^{2+} -permeable AMPA receptors

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POSTSYNAPTIC Ca^{2+} elevation during synaptic transmission is an important trigger for short- and long-term changes in synaptic strength in the vertebrate central nervous system¹. The AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors, a subfamily of glutamate receptors, mediate much of the excitatory synaptic transmission in the brain and spinal cord². It has been shown that a subtype of the AMPA receptor is Ca^{2+} -permeable^{3–6} and is present in subpopulations of neurons^{7–12}. When synaptically localized¹³, these receptors should mediate postsynaptic Ca^{2+} influx, providing a trigger for changes in synaptic strength. Here we show that Ca^{2+} -permeable AMPA receptors are synaptically localized on a subpopulation of dorsal horn neurons, that they provide a synaptically gated route of Ca^{2+} entry, and that activation of these receptors strengthens synaptic transmission mediated by AMPA receptors. This pathway for postsynaptic Ca^{2+} influx may provide a new form of activity-dependent modulation of synaptic strength.

Fast application of a hyperosmotic solution to dorsal horn neurons in culture caused a large increase in the probability of transmitter release, as indicated by an enhancement in the frequency of miniature excitatory postsynaptic currents (mEPSCs; Fig. 1a) and shown previously at several types of synapses^{14–17}. The mEPSCs in our studies were mediated by AMPA receptors because they were blocked by 25 μM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) (Fig. 1a). The enhanced release of neurotransmitter was retained in a low- Ca^{2+} hyperosmotic bath (Fig. 1a). Synaptic Ca^{2+} transients were evoked by hyperosmotic solution in the presence of 25 μM D-APV (D-2-amino-5-phosphonopivalic acid) and were blocked by 25 μM CNQX (Fig. 1b), indicating that they were due to Ca^{2+} entry through synaptically gated Ca^{2+} -permeable AMPA receptors. The Ca^{2+} transients required Ca^{2+} influx and were not due solely to release of Ca^{2+} from intracellular stores¹⁸, because a hyperosmotic bath with no added Ca^{2+} did not evoke Ca^{2+} transients (Fig. 1b). These results demonstrate the synaptic localization of Ca^{2+} -permeable AMPA receptors on dorsal horn neurons in culture and show that their activation leads to an increase in postsynaptic intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) in neurites.

JSTX-3, a synthetic derivative from the venom of the spider *Nephila clavata*¹⁹, selectively inhibits recombinant AMPA receptors lacking the GluR2 subunit²⁰, a molecular determinant whose

absence is required for Ca^{2+} permeability of the AMPA receptors^{4–6}. We tested the ability of the use-dependent JSTX-3 (ref. 20) to select among natively expressed AMPA receptors on neurons that express different combinations of Ca^{2+} -permeable and Ca^{2+} -impermeable AMPA receptors²¹. The relative level of expression of Ca^{2+} -permeable AMPA receptors was determined by the

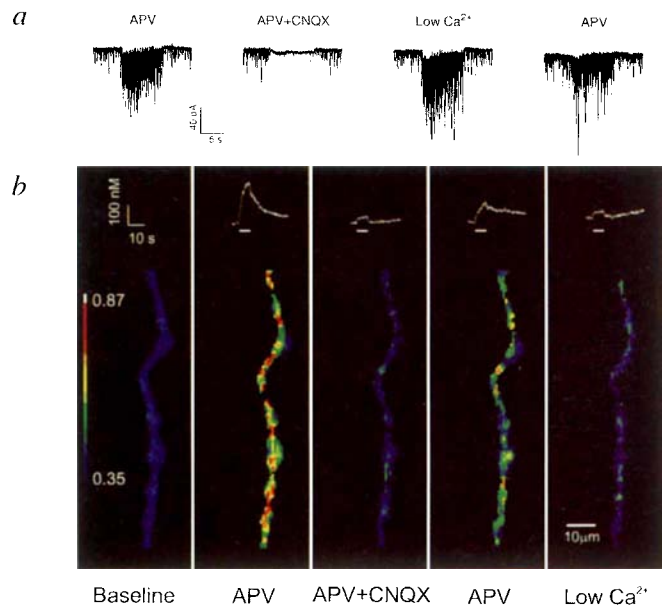


FIG. 1 Hyperosmotic solution transiently promotes transmitter release in a Ca^{2+} -independent manner (a), enhancing Ca^{2+} influx through postsynaptic Ca^{2+} -permeable AMPA receptors (b); a and b were recorded from different cells. At least 2 min were allowed between applications to permit replenishing of the synaptic vesicle pool. a, CNQX quickly and effectively blocked mEPSCs when applied with hyperosmotic solution, which enhanced mEPSC frequency even in low external $[\text{Ca}^{2+}]$. b, Fura-2 ratio images of a neurite to which hyperosmotic solution was applied in the presence of the indicated drugs. Insets, traces of $[\text{Ca}^{2+}]_i$ against time for each of the conditions represented by the images. Bars under the traces represent the application time of the hyperosmotic solutions. The artefact present in all traces may be due to optical perturbation, caused by application of the hyperosmotic solution. Minimum and maximum ratios in the colour scale correspond, respectively, to 1 nM and 450 nM Ca^{2+} . The baseline $[\text{Ca}^{2+}]_i$ increased from 50–70 nM between the first and last application of hyperosmotic solution. The average change in $[\text{Ca}^{2+}]_i$ evoked by hyperosmotic solution with D-APV when measured over a large section of neurite was 95 ± 79 nM ($n = 3$). This Ca^{2+} elevation was blocked by $95 \pm 8\%$ by 25 μM CNQX. In a similar series of experiments using hyperosmotic solution with 75 mM CaCl_2 and D-APV, the change in $[\text{Ca}^{2+}]_i$ was 28 ± 20 nM, which was inhibited $86 \pm 13\%$ by CNQX ($n = 3$).

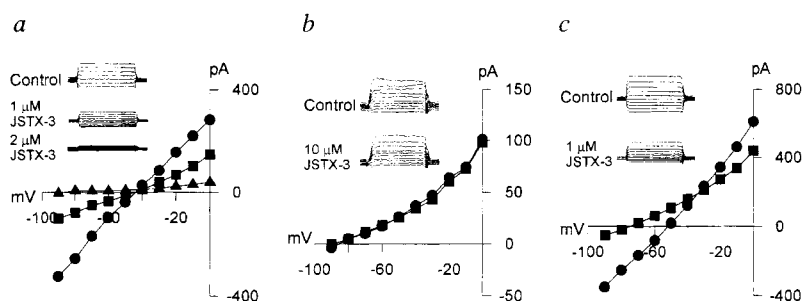
METHODS. Spinal-cord dorsal-horn neuron cultures were prepared as described¹⁰ and used between 2–5 weeks. Cells were perfused with bath solution containing (in mM) 145 NaCl, 5 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, 5.5 D-glucose and 5×10^{-4} tetrodotoxin (TTX) (pH 7.3, 325 mOsm, at room temperature). Hyperosmotic solution was bath solution with osmolality adjusted to 530 mOsm with sucrose, plus 30 μM LaCl_3 to block all voltage-gated Ca^{2+} channels¹⁰, plus 3 mM CaCl_2 (5 mM CaCl_2 total) and 25 μM D-APV. Low- Ca^{2+} hyperosmotic solution did not have CaCl_2 or D-APV. Test and wash solutions were applied through a fast perfusion system. For a, neurons were patch-clamped at -70 mV in perforated patch configuration using Cs⁺-filled electrodes²⁶. For b, neurons were loaded with 1 mM Fura-2 pentapotassium salt²⁷ through a potassium gluconate patch pipette in the whole-cell configuration. After 10 min of dye loading, the pipette was withdrawn and the dye allowed to diffuse into the neurites for an additional 20 min. Background-subtracted ratio images were obtained by the single-wavelength ratio method²⁸, using 340 or 360 nm as the reference wavelength and 380 nm as the measured wavelength. Each panel in b is 1 image frame taken at the peak of the response, with an average baseline subtracted for each condition.

apparent reversal potential (E_{rev}) of currents evoked by 100 μ M kainate in a Na^+ -free bath with 10 mM Ca^{2+} ($0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath)²¹ (Fig. 2 legend). Figure 2a shows data from a cell expressing large numbers of Ca^{2+} -permeable AMPA receptors, as indicated by an E_{rev} near -40 mV. The amplitude of the kainate-evoked current was strongly decreased, with little change in E_{rev} , when neurons were treated with 1 μ M JSTX-3 plus 100 μ M kainate. The currents were almost completely blocked by subsequent exposure to 2 μ M JSTX-3 plus kainate. Figure 2b shows data from a cell expressing few Ca^{2+} -permeable AMPA receptors, as indicated by an E_{rev} near -90 mV. Little block was evident following 1 μ M ($n = 3$; not shown) or 10 μ M ($n = 4$) JSTX-3 except for a slight negative shift in the E_{rev} . Figure 2c shows data from a cell

expressing a mixture of both Ca^{2+} -permeable and Ca^{2+} -impermeable AMPA receptors (E_{rev} near -60 mV). JSTX-3 shifted the E_{rev} to a more negative value and decreased current amplitude, presumably by decreasing the contribution of the Ca^{2+} -permeable channels to the total AMPA receptor population. These results show that E_{rev} in a $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath is a good indicator of the relative proportion of Ca^{2+} -permeable AMPA receptor expressed on each cell and that JSTX-3 block is selective for native Ca^{2+} -permeable AMPA receptors.

If Ca^{2+} -permeable AMPA receptors participate in synaptic transmission, the amplitudes of mEPSCs should decrease in the presence of JSTX-3. The data shown in Fig. 3a were recorded from a cell expressing moderate numbers of Ca^{2+} -permeable

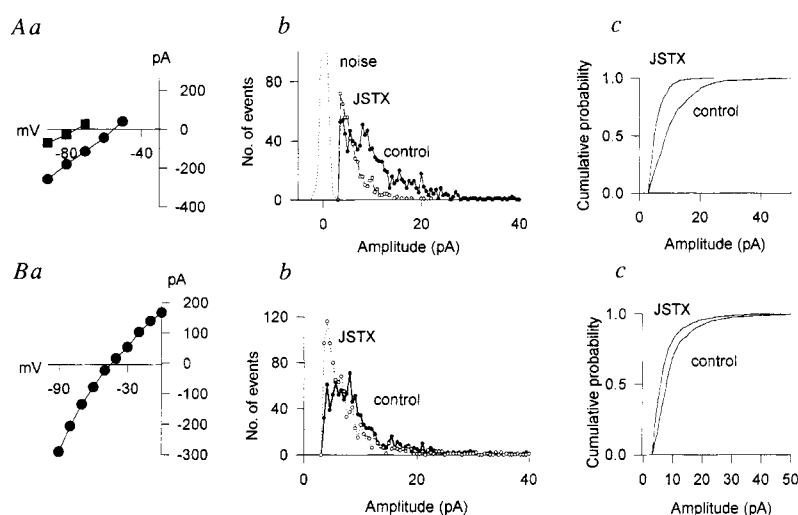
FIG. 2 JSTX-3 block is selective for native Ca^{2+} -permeable AMPA receptors. Currents were evoked by 100 μ M kainate in a $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath at different membrane potentials²¹ before and after exposure to JSTX-3. Insets show raw traces of the currents from which the corresponding I - V curves are constructed. a, Data from a neuron expressing high numbers of Ca^{2+} -permeable AMPA receptors. The amplitudes of kainate-evoked currents are plotted before (●) and after block with 1 μ M (■), or 2 μ M JSTX-3 (▲). For all cells of this group with an E_{rev} near -40 mV (-39.3 ± 5.1 mV; $n = 3$, mean $\pm$ s.d.), the apparent Ca^{2+} permeability, $P_{\text{Ca}^{2+}}/P_{\text{Cs}^+}$ (ref. 22) was 1.14 ± 0.28 . The kainate-evoked current was decreased by $\sim 70\%$ ($71.3\% \pm 11.5\%$) by 1 μ M JSTX-3, with no change in E_{rev} . b, 10 μ M JSTX-3 has no effect on Ca^{2+} -impermeable AMPA receptors, as can be seen by comparing kainate-evoked current amplitudes under control conditions (●) and after addition of 10 μ M JSTX-3 (■). For cells of this group with an E_{rev} near -90 mV (-84.3 ± 8.5 mV; $n = 7$), the $P_{\text{Ca}^{2+}}/P_{\text{Cs}^+}$ value for AMPA receptors was 0.18 ± 0.06 ($n = 7$). c, Effect of JSTX-3 on a cell expressing both Ca^{2+} -permeable and Ca^{2+} -impermeable AMPA receptors. Kainate-evoked currents are shown under control conditions (●) and after addition of 1 μ M JSTX-3 (■). For cells of this group with an E_{rev} between -40 and -70 mV (-62.3 ± 8.1 mV; $n = 6$), $P_{\text{Ca}^{2+}}/P_{\text{Cs}^+}$ was 0.43 ± 0.14 . 1 μ M JSTX-3 decreased current amplitude and shifted the E_{rev} to a more negative value (-84.0 ± 20.5 mV; $n = 6$) and the $P_{\text{Ca}^{2+}}/P_{\text{Cs}^+}$ to 0.22 ± 0.15 . METHODS. Neurons were voltage-clamped at -70 mV in perforated-patch configuration with Cs^+ filled electrodes²⁶. Bath solution also contained 10 μ M bicuculline, 5 μ M strychnine and 25 μ M D-APV. $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath was composed of (in mM): 10 CaCl_2 , 155 NMDG (*N*-methyl-D-glucamine),



10 HEPES, 5.5 D-glucose and 5×10^{-4} TTX (pH 7.3, 315–325 mOsm). The $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath was preapplied to the cell for 1 s followed by 100 μ M kainate in the same bath for 2 s. Voltage steps were applied for 4 s starting from the time when $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath was on and ending when $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath was off. After obtaining control data, each cell was exposed to 1–10 μ M JSTX-3 plus 100 μ M kainate in $0 \text{ Na}^+/0 \text{ Ca}^{2+}$ (with 20 mM CsCl added) bath for 4 min. Each preparation was subsequently washed for 2 min with normal bath and a new I - V curve for kainate-evoked currents was determined. JSTX-3 was added with kainate because its block is use-dependent²⁰. Application of 100 μ M kainate in $0 \text{ Na}^+/0 \text{ Ca}^{2+}/20 \text{ Cs}^+$ bath solution without JSTX-3 produced no significant change in E_{rev} or current amplitude in the 6 cells tested. This bath condition avoided loading the cells with Ca^{2+} during kainate application but allowed JSTX-3 access to binding sites in the open channel. Kainate-evoked currents in rat dorsal horn neurons are predominantly mediated by AMPA receptors²¹.

FIG. 3 Synaptic transmission through Ca^{2+} -permeable AMPA receptors is revealed by JSTX-3. a, a, The I - V curve is shown for kainate currents evoked in $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath before (●) and after (■) exposure to 10 μ M JSTX-3 plus 100 μ M kainate for 4 min (see Methods). b, mEPSCs were recorded from the same cell for 5 min before (control) and after exposure to JSTX-3. Amplitudes were measured and plotted in histogram form. The noise curve shows the baseline noise amplitude measured 64 ms before each mEPSC. c, The same data as in b are plotted in a cumulative histogram. B, a, A neuron shows high expression levels of Ca^{2+} -permeable AMPA receptors as indicated by E_{rev} near -45 mV for currents evoked by 100 μ M kainate in a $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath. b, Amplitude histograms show mEPSCs amplitude distribution before (control) and after exposure to JSTX-3 alone. c, Cumulative probability amplitude histograms of 1,000 mEPSCs recorded from the same cell before and after application of JSTX-3.

METHODS. mEPSCs were recorded from neurons voltage-clamped at -70 mV in perforated patch configuration. Records were filtered at 1 kHz and sampled at 3.3 kHz. The threshold for detection of mEPSCs was set at -3 or -4 pA. Events showing summation with previous events were not included in the amplitude analysis. For 6 cells, including that in a, the E_{rev} was determined as for Fig. 2, before recording mEPSCs for 5 min. Cells were exposed to 10 μ M JSTX-3 and 100 μ M kainate in $0 \text{ Na}^+/0 \text{ Ca}^{2+}/20 \text{ Cs}^+$ bath for 4 min, washed and then 5 min of mEPSCs were recorded followed by



measurement of E_{rev} . In 2 cells, JSTX-3 block was partially reversed by repeatedly stepping the membrane potential to $+100$ mV in the presence of 100 μ M kainate for 2 s. Control applications of kainate alone in $0 \text{ Na}^+/0 \text{ Ca}^{2+}/20 \text{ Cs}^+$ bath produced no change in mEPSC amplitudes.

AMPA receptors, as indicated by a E_{rev} near -55 mV (Fig. 3A, a). mEPSCs were recorded in a normal bath for 5 min before and 5 min after a 4-min exposure to $10 \mu\text{M}$ JSTX-3 and $100 \mu\text{M}$ kainate. In this and three other cells with moderate to high levels of Ca^{2+} -permeable AMPA receptors ($E_{rev} = -49 \pm 3.9$ mV), mean mEPSC amplitude was significantly decreased from 17 ± 5 to 7.8 ± 2.4 pA by JSTX-3 ($P < 0.05$), indicating a strong contribution of Ca^{2+} -permeable AMPA receptors to the synaptic response (Fig. 3A, b and c). A substantial decline in mEPSC frequency was also seen after treatment with JSTX-3 (Fig. 3A, b). We attribute that, at least in part, to the reduction in some mEPSC amplitudes to a level below our threshold of detection. The mean E_{rev} in these cells following exposure to JSTX-3 plus kainate became -79 ± 2.3 mV, indicating a strong block of many of the Ca^{2+} -permeable AMPA receptors by this protocol (Fig. 3A, a). In three cells with few Ca^{2+} -permeable AMPA receptors ($E_{rev} = -95 \pm 9$ mV), JSTX-3 had no significant effect on mEPSC amplitude (8.2 ± 0.7 and 7.2 ± 1.1 pA, before and after exposure to JSTX-3, respectively). These data indicate that Ca^{2+} -permeable AMPA receptors account for more than half of the synaptically localized AMPA receptors on a subpopulation of dorsal horn neurons in culture.

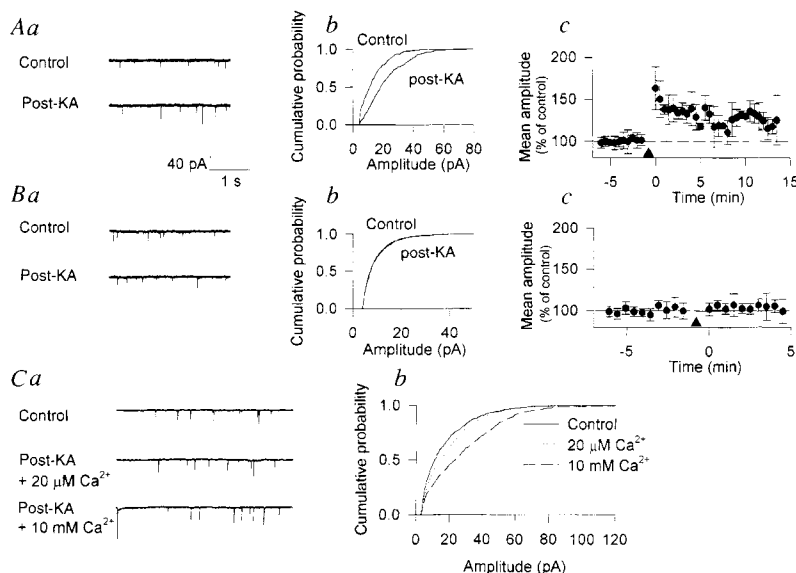
Figure 3B shows results from an experiment in which only the synaptically released glutamate opened AMPA receptor channels to allow use-dependent block by JSTX-3, a condition unfavourable for use-dependent block to develop but potentially less likely to produce kainate-dependent receptor modulation. After exposing a cell with high levels of Ca^{2+} -permeable AMPA receptors to JSTX-3 alone for 20 min, a clear decrease in the average mEPSC amplitude was observed (Fig. 3B, b and c). For cells of this group ($E_{rev} = -47 \pm 15$ mV, $n = 9$), the average mEPSC amplitude was -13.9 ± 2.4 pA before applying $10 \mu\text{M}$ JSTX-3 and was significantly decreased to 11.6 ± 2.3 pA after JSTX-3 ($P < 0.01$, paired t -test). For cells expressing few Ca^{2+} -permeable AMPA receptors, ($E_{rev} = -76 \pm 8$, $n = 6$), the average mEPSC amplitude before and after exposure to JSTX-3 was -11 ± 5.7 pA and -11 ± 6.1 pA, respectively.

NMDA receptor-dependent changes in mEPSC amplitude and

frequency have yielded conflicting results in paradigms of synaptic potentiation in hippocampal neurons^{16,17}. Ca^{2+} entry through voltage-gated Ca^{2+} evoked a transient enhancement of AMPA receptor function lasting for tens of minutes²², as tested by agonist application as well as mEPSC amplitude. To test whether Ca^{2+} entry through Ca^{2+} -permeable AMPA receptors affects synaptic strength, we measured changes of mEPSC amplitudes after application of kainate to dorsal horn neurons with high or low expression levels of Ca^{2+} -permeable AMPA receptors. Figure 4A shows data from an experiment conducted on a neuron with high levels of Ca^{2+} -permeable AMPA receptors ($E_{rev} = -42$ mV). A significant increase in mEPSC amplitude that lasted for more than 5 min was observed after kainate application (Fig. 4A, c). The cumulative amplitude histogram (Fig. 4A, b) shows a significant shift to the right ($P < 0.01$; Kolmogorov-Smirnov test). For all cells tested expressing relatively high levels of Ca^{2+} -permeable AMPA receptors, the average mEPSC amplitude was -13.71 ± 3.93 pA at 5 min before kainate application and -18.02 ± 4.93 pA ($n = 8$) at 5 min after kainate ($P < 0.01$, paired t -test). When mEPSC amplitude was plotted as a function of time for these cells, a significant potentiation of mEPSC amplitude was seen over the first 5 min following kainate application (Fig. 4A, c). Two cells showed significant elevation of mEPSC amplitude for up to 15 min, the longest period tested. For the other cells, mEPSCs decayed gradually back to control. The potentiation was dependent on Ca^{2+} entry as no potentiation was observed when kainate was applied in $20 \mu\text{M}$ Ca^{2+} solution ($n = 3$), whereas subsequent kainate application in 10 mM Ca^{2+} induced potentiation (Fig. 4C, a and b). No consistent change in mEPSC frequency was observed in these experiments.

To confirm that mEPSC potentiation is due to activation of Ca^{2+} -permeable AMPA receptors rather than to activation of voltage-gated Ca^{2+} channels caused by poor space clamp control²³ or to other effects related to kainate application, we conducted similar experiments on cells that expressed low levels of Ca^{2+} -permeable AMPA receptors (Fig. 4B, a–c). For all cells of this group, the average mEPSC amplitude before and 5 min after kainate applications was -9.81 ± 3.32 pA and -10.07 ± 3.03 pA

FIG. 4 mEPSC amplitude is enhanced after Ca^{2+} entry through Ca^{2+} -permeable AMPA receptors. A, a, Sample traces of mEPSCs in a cell with high expression of Ca^{2+} -permeable AMPA receptors before (top) and after (bottom) potentiation. mEPSC amplitudes were enhanced after repeated but brief activation of Ca^{2+} -permeable AMPA receptors by 1 mM kainate in the $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath. b, Cumulative probability amplitude histograms of mEPSCs collected over 5 min before and 5 min after kainate pulses. Measurements are from the same cell as in a. c, Time course of potentiation by kainate from 8 cells with high expression of Ca^{2+} -permeable AMPA receptors ($E_{rev} = -41.5 \pm 8.2$ mV; $n = 8$). mEPSC amplitude is normalized to the average mEPSC value during 5 min before kainate pulses. B, a, Sample traces from a cell with few Ca^{2+} -permeable AMPA receptors before and after kainate application using the same protocol as in A. b, Cumulative probability amplitude histograms of mEPSCs collected over 5 min before and after kainate pulses. Data are measurements from the same cell as in a. c, Normalized average amplitude of mEPSCs in 5 cells with few Ca^{2+} -permeable AMPA receptors ($E_{rev} = -70.2 \pm 6.8$ mV; $n = 5$) shows no potentiation. C, a, Sample traces of mEPSCs in a cell with high expression of Ca^{2+} -permeable AMPA receptors before (control) and after potentiation in bath with $20 \mu\text{M}$ Ca^{2+} , and bath with 10 mM Ca^{2+} as in A, b and B, b. b, Cumulative probability amplitude histograms of mEPSCs collected over 5 min before and after kainate pulses. METHODS. mEPSCs were recorded as for Fig. 3. Control mEPSCs were sampled in standard bath solution for at least 5 min. 1 mM kainate was then applied 10 times to the whole cell in a $0 \text{ Na}^+/10 \text{ Ca}^{2+}$ bath solution for 0.5-s at 10-s intervals. The cell was quickly returned to standard bath solution and



mEPSCs were sampled again for over 15 min. The bath conditions for kainate application were chosen to optimize Ca^{2+} entry through AMPA receptors while minimizing possible activation of voltage-gated Ca^{2+} channels that could occur as a result of voltage escape²⁶. E_{rev} was determined at the end of the experiment.

($n = 5$), respectively (not significant; $P = 0.19$, paired t -test). This strongly suggests that the synaptic potentiation in Fig. 4A, C was a result of Ca^{2+} entry through Ca^{2+} -permeable AMPA receptors.

One of the most prominent distinctions between Ca^{2+} -permeable AMPA receptors and the more well known Ca^{2+} -permeable glutamate receptors, the NMDA receptors, is that the latter are subject to a voltage-dependent block by Mg^{2+} , whereas Ca^{2+} -permeable AMPA receptors are not. Consequently, the synaptic Ca^{2+} fluxes associated with activation of Ca^{2+} -permeable AMPA receptors will be prominent at negative membrane potentials where the driving force on Ca^{2+} is high²⁴, whereas depolarized membrane potentials will favour strong Ca^{2+} fluxes through NMDA receptors^{24,25}. Thus synaptic Ca^{2+} -permeable AMPA receptors may be expected to affect synaptic strength with a different sensitivity to activity than NMDA receptors. The synaptic strengthening that occurs following Ca^{2+} entry through Ca^{2+} -permeable AMPA receptors suggests that these channels are likely to provide an important physiological signal for triggering changes in synaptic transmission. □

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Transduction of bitter and sweet taste by gustducin

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SEVERAL lines of evidence suggest that both sweet and bitter tastes are transduced via receptors coupled to heterotrimeric guanine-nucleotide-binding proteins (G proteins) (reviewed in refs 1, 2). Gustducin is a taste receptor cell (TRC)-specific G protein that is closely related to the transducins³. Gustducin and rod transducin, which is also expressed in TRCs (ref. 4), have been proposed to couple bitter-responsive receptors to TRC-specific phosphodiesterases to regulate intracellular cyclic nucleotides^{2–5}. Here we investigate gustducin's role in taste transduction by generating and characterizing mice deficient in the gustducin α -subunit (α -gustducin). As predicted, the mutant mice showed reduced behavioural and electrophysiological responses to bitter compounds, whereas they were indistinguishable from wild-type controls in their responses to salty and sour stimuli. Unexpectedly, mutant mice also exhibited reduced behavioural and electrophysiological responses to sweet compounds. Our results suggest that gustducin is a principal mediator of both bitter and sweet signal transduction.

Gene replacement was used to generate a null mutation of the α -gustducin gene. The murine α -gustducin gene was cloned and sequences surrounding the first protein coding exon were used to create the targeting vector (Fig. 1a). Positive/negative selection⁶ was used to enrich for embryonic stem (ES) cell clones with a homologously recombined α -gustducin allele (Fig. 1). Chimaeric mice, generated from these ES cells by blastocyst injection, were back-crossed to C57BL/6J mice. Homozygous mice harbouring the recombined gustducin allele, genetically (C57BL/6J $\times$ 129/SvEmsJ) F_2 , were produced by intercrossing heterozygous animals (Fig. 1b). Heterozygous and homozygous null mice were viable, healthy and fertile.

Taste epithelia from homozygous null mice were morphologically indistinguishable from epithelia of wild-type littermates (Fig. 1c–f). Mice have three types of taste papillae: fungiform, scattered throughout the anterior two thirds of the tongue; foliate, in lateral grooves; and a single circumvallate, at the back of the tongue⁷. The null mice had all three types of taste papillae, with an appropriate number of taste buds in each papilla and a normal complement of TRCs per bud.

α -Gustducin messenger RNA is normally expressed in TRCs of circumvallate, foliate and fungiform taste papillae³. However, in the null mice, α -gustducin expression was not detectable in the TRCs of circumvallate (compare Fig. 1c and e), foliate or fungiform taste papillae (data not shown). The sense probe controls (Fig. 1d,f) showed no hybridization to lingual tissue. We conclude that the targeting event resulted in a null allele of α -gustducin.

Forty-eight-hour two-bottle preference tests⁸ were used to compare the taste responses of α -gustducin null mice with those of their wild-type siblings. A preference ratio (tastant solution consumed as a fraction of total liquid consumed) was calculated for each animal at each concentration. Tastants that are primarily salty (NaCl), sour (HCl), bitter (denatonium benzoate and quinine sulphate) or sweet (sucrose and the highly potent guanidine sweetener SC45647) to humans were tested. Two-factor (strain $\times$ concentration) analyses of variance (ANOVAs) (Table 1) were used to determine whether wild-type and null mice differed in their behavioural responses to tastants.

Responses of null mice to a concentration series of NaCl were similar to those of wild-type mice. Likewise, no strain difference was evident in the responses to a range of HCl solutions (Table 1; Fig. 2a, b). In both cases, all mice were indifferent to initial (low) tastant concentrations and exhibited aversive responses to higher concentrations. These data demonstrate that salty and sour behavioural responses are unaffected by the absence of α -gustducin.

In contrast, aversive responses to two bitter substances, denatonium benzoate and quinine sulphate, were diminished in the null mice compared to wild-type siblings (Table 1; Fig. 2c, d). Wild-type mice began to avoid denatonium at the 100 μM concentration, whereas null mice remained indifferent to denatonium even at 1 mM. The null mice required a ~ 40 -fold higher concentration of denatonium and a ~ 100 -fold higher concentration of

quinine than did their wild-type siblings to elicit comparable aversive responses. These data demonstrate that avoidance responses to these two bitter compounds are profoundly diminished in the absence of α -gustducin.

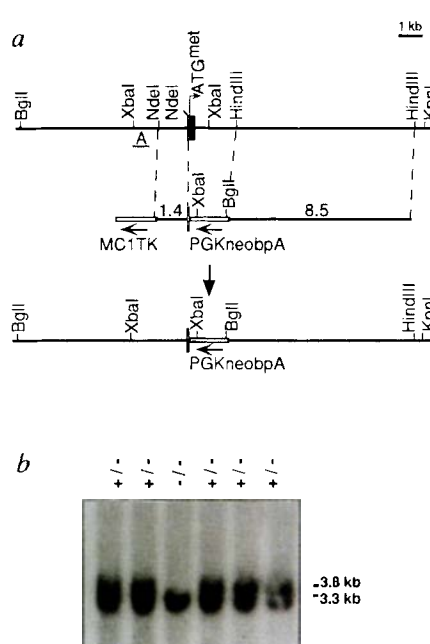
Unexpectedly, preference responses to two sweet substances, sucrose (Fig. 2e) and the sweetener SC45647 (ref. 9) (Fig. 2f) were reduced in the null mice compared to wild-type siblings (Table 1). Wild-type mice preferred sucrose at ≥ 50 mM concentrations. However, the null mice remained indifferent at sucrose concentrations below 500 mM and required a ~ 17 -fold higher concentration of sucrose than did their wild-type siblings to elicit comparable preference responses. Similar strain differences were found with SC45647 (Fig. 2f). Wild-type mice preferred SC45647 at 0.1 mM and 0.5 mM, whereas null mice remained indifferent at both of these concentrations. Additional testing indicated that the null mice preferred 2 mM SC45647 (preference ratio = 0.71; data not shown). We conclude that preference responses to these two sweet compounds are profoundly diminished in the absence of α -gustducin.

To confirm that the behavioural deficit in the mutants was due

to a peripheral taste defect, we performed recordings from the chorda tympani branch of the facial (VII) nerve in null and wild-type mice. The chorda tympani innervates the anterior tongue and is responsive to salty, sweet, sour and bitter stimuli¹⁰. Summated chorda tympani responses were recorded during lingual stimulation with NaCl, HCl, quinine sulphate, denatonium benzoate, sucrose and SC45647 (sample traces shown, Fig. 3a, d, g). Each response was quantified by measuring two parameters: (1) the maximum amplitude of the neural response within four seconds of stimulus onset (phasic response), and (2) the response magnitude occurring ten seconds after stimulus onset (tonic response). The phasic response represents the initial nerve response to tastant stimulation of TRCs. The tonic response represents the sustained nerve response to continuous tastant stimulation of TRCs. Chorda tympani responses were normalized to the mean phasic response to NH_4Cl ; only normalized phasic responses are shown (Fig. 3b, c, e, f, h, i). Two-factor (strain $\times$ concentration) ANOVAs were used to detect differences in the chorda tympani responses of null and wild-type mice (Table 1).

Both phasic and tonic responses of null mice were similar to

FIG. 1 Generation of α -gustducin null mice. **a**, Top, Map of the murine α -gustducin gene showing the first exon (open/filled box): 5' untranslated leader sequence (open box), coding region for first 18 amino acids (filled box), and initiation codon (ATG^{met}). The probe used to screen G418-resistant ES cell clones is indicated (line A below map). Middle, map of the targeting vector, indicating the MC1tk and PGKneobpA genes. The direction of transcription is indicated. The gustducin-homologous 5' region is a 1.4-kb *NdeI* fragment which includes the transcription initiation sequences and 30 nucleotides (nt) of untranslated leader sequence from exon 1 (open box). The 3' homologous region is an 8.5-kb *HindIII* fragment derived from the first intron. Bottom, map of the predicted structure of the homologously recombined gustducin allele, showing the presence of the first exon (open box) immediately preceding the PGKneo gene.



b, Genomic analysis of offspring from a (+/-) $\times$ (+/-) mating. *XbaI*-digested genomic DNAs purified from tail biopsies were screened with probe A (see **a**). The wild-type α -gustducin restriction fragment is 3.8 kb. The targeting event generates a novel 3.3-kb fragment which includes a portion of the first exon fused to the *neo* gene. The third lane (-/-) identifies a homozygous null mouse. **c-f**, Photomicrographs of frozen sections of mouse taste papillae hybridized with ^{33}P -labelled murine gustducin probe and counterstained with haematoxylin-eosin. Bright-field (upper panels) and dark-field (lower panels) images are shown of antisense probe hybridized to circumvallate papillae from wild-type (**c**) and α -gustducin null mice (**e**), and of control sense probe hybridized to circumvallate papillae from wild-type (**d**) and α -gustducin null mice (**f**).

METHODS. The murine α -gustducin gene was cloned from a 129/Sv mouse genomic lambda library (Lamda FIX II, Stratagene) using rat α -gustducin cDNA³ as probe. One clone, $\lambda 4$, which contained the first protein-coding exon, was mapped and partially sequenced. A 1.4-kb *NdeI* fragment containing sequences homologous with the 5'-most sequence of the rat α -gustducin cDNA and an 8.5-kb *HindIII* fragment derived from the first intron were subcloned into a *neo/tk* selection vector. The resulting targeting vector DNA was linearized with *Sall* before electroporation. Positive/negative selection⁶ was used to obtain an allele of α -gustducin in which the first protein coding region (including the ATG initiation codon, and the splice donor sequences) was replaced with the PGK-*neo* gene²² oriented opposite to gustducin's direction of transcription. The targeting vector also included the herpes simplex virus thymidine kinase (*tk*) gene for negative selection²³.

Transformation of the ES cell line W9.5 (ref. 24) was done as described²⁵ using *Sall*-linearized α -gustducin targeting vector. Electroporation, selection and expansion of G418- and FIAU-resistant ES cell colonies were done as described²⁶. ES cell and tail biopsy DNAs were prepared as described²⁷. DNA (~ 5 μg) was digested with *XbaI*, electrophoresed and electroblotted as described²⁵. Membranes were hybridized as described²⁵ with a radiolabelled 0.7-kb *BglII*-*XbaI* α -gustducin probe (probe A), consisting of unique sequences (data not shown) upstream from the 5'-most sequences present in the targeting vector. Southern analysis of genomic DNA from clonally expanded ES cell colonies indicated a homologous recombination frequency of 7%. ES cell clones containing a targeted gustducin allele were then karyotyped. Two independent clones with 40XY karyotypes were used to produce chimaeric mice by blastocyst injection²⁵. Chimaeric males were bred to C57BL/6J females. Heterozygous progeny containing the mutant α -gustducin allele were intercrossed. Both lines yielded homozygous α -gustducin null pups with $\sim 25\%$ frequency. We used male mice (both -/- and +/-) with $\sim 50/50$ mix of 129/SvEMsJ and C57BL/6J backgrounds. For expression analysis, T3 and T7 RNA polymerases were used to generate ^{33}P -labelled RNA probes²⁸ from mouse gustducin exon-8 sequences subcloned into pBluescriptII. Transcription reactions containing 250 μCi ^{33}P -labelled thio-UTP were done according to the manufacturer's instructions. (Promega). Frozen sections (10 μm) of wild-type or α -gustducin null mouse tongues were hybridized with RNA probes (1×10^6 c.p.m. per section). Slides were processed as described^{32,29}.

those of wild-type mice for a concentration series of NaCl (Table 1; Fig. 3b). Similarly, no differences were seen with HCl solutions (Table 1; Fig. 3c). The electrophysiological results with NaCl and HCl are consistent with the behavioural results (Fig. 2) and indicate that α -gustducin null mice are unaltered in their ability to detect salty and sour stimuli. In contrast, but again consistent with the behavioural data, they showed reduced electrophysiological responses to bitter substances; compared to normal controls, mutants showed reduced responses to both denatonium benzoate and quinine, apparent in both phasic and tonic responses (Table 1; Fig. 3e, f). Similarly, the electrophysiological responses to the sweet compounds sucrose and SC45647 were significantly lower than those of wild-type mice (Table 1; Fig. 3h, i). Indeed, the mutant mice showed almost no electrophysiological response to either compound, even at concentrations that are intensely sweet to humans.

Taken together, our behavioural and electrophysiological results indicate that α -gustducin is a principal mediator of bitter and sweet taste transduction, although it is not involved in salty or sour transduction. At high concentrations, however, α -gustducin-deficient mice show a residual behavioural response to both bitter and sweet compounds, suggesting that additional G proteins may also be involved (candidate G proteins expressed in TRCs include

G_s, G_{i-3}, G₁₄ and rod transducin). Non-gustatory factors may also contribute to these behavioural responses. Interestingly, although the effect of the null mutation on the chorda tympani response was greater for sweet than for bitter compounds, the behavioural effect was greater with bitter compounds. Behavioural responses to bitter compounds may depend more on the glossopharyngeal nerve than on the chorda tympani, because the former contains a larger proportion of bitter-specific fibres¹¹. Consistent with this idea, preliminary glossopharyngeal nerve recordings demonstrated profoundly diminished responses of the null mice to quinine sulphate and denatonium benzoate (K.S.G., Y. Ninomiya, G.T.W. and R.F.M., unpublished).

Several lines of evidence implicate G proteins and their coupled receptors in the transduction of bitter and sweet tastes (reviewed in refs 1, 2). Denatonium increases inositol trisphosphate levels in mouse taste tissue¹² and leads to the release of intracellular Ca²⁺ from rat TRCs (ref. 13). Bitter transduction may involve activation of phospholipase C by gustducin's $\beta\gamma$ subunits and/or by an α_q -like subunit (α_{14} for example). Sucrose causes a GTP-dependent increase in taste tissue cAMP, apparently by activating adenylyl cyclase¹⁴⁻¹⁶. Microinjection of cyclic AMP into TRCs (ref. 17) or the addition of membrane permeant cyclic nucleotides^{18,19} leads to inactivation of K⁺ channels and TRC depolarization. Genetic

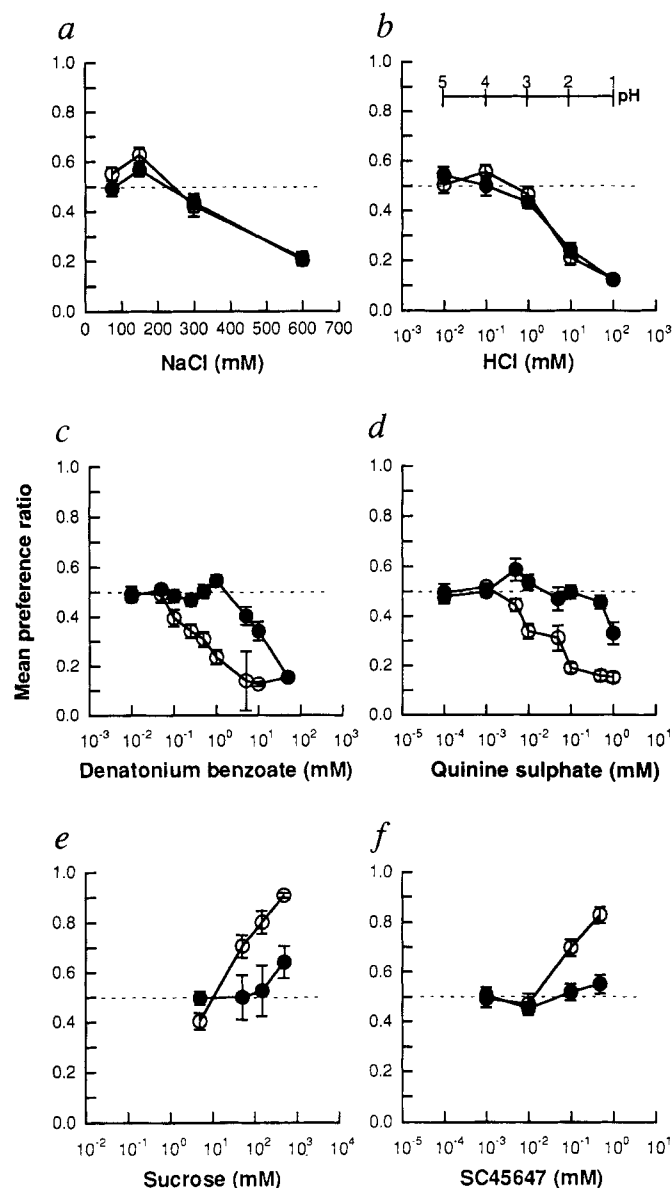


FIG. 2 Mean preference ratios from 48-h two-bottle (tastant versus distilled water) preference tests of male wild-type (open circles) and null (filled circles) mice. Tastant concentrations are listed in order of presentation. a, Behavioural responses to NaCl (75, 150, 300 and 600 mM) of wild-type mice ($n = 11$) and their null siblings ($n = 11$). No significant strain or interaction effects were found (P values were >0.05 ; Table 1). b, Behavioural responses to HCl (0.01, 0.1, 10 and 100 mM; pH range of 5.0 to 1.0) of wild-type mice ($n = 10-12$) and their null siblings ($n = 10-12$). No significant strain or interaction effects were found (P values were >0.05 ; Table 1). c, Behavioural responses to denatonium benzoate (0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0, 50.0 mM) of wild-type mice ($n = 10-11$) and their null siblings ($n = 10-11$). Results of t -tests are as follows: 0.25 mM ($P < 0.01$); 0.5–10.0 mM ($P < 0.0001$). d, Behavioural responses to quinine sulphate (0.1, 1.0, 5.0, 10.0, 50.0, 100.0 μ M; 0.5, 1.0 mM) of wild-type mice ($n = 9-12$) and their null siblings ($n = 10-12$). Results of t -tests are as follows: 5.0, 50.0 μ M, 1.0 mM ($P < 0.05$); 10.0, 100, 500 μ M ($P < 0.01$). e, Behavioural responses to sucrose (5, 50, 150 and 500 mM) of wild-type mice ($n = 7-12$) and their null siblings ($n = 11-12$). Results of t -tests are as follows: 50–500 mM ($P < 0.001$). f, Behavioural responses to SC45647 (1, 10, 100 and 500 μ M) of wild-type mice ($n = 10-12$) and their null siblings ($n = 10-12$). Results of t -tests are as follows: 100 and 500 μ M ($P < 0.001$).

METHODS. Polymerase chain reaction (PCR) was used to analyse male mice from heterozygous sib matings. Wild-type (+/+) and α -gustducin null (–/–) male siblings were used for behavioural tests. Both wild-type and null mice were genetically (C57BL/6J $\times$ 129/SvEms)F₂. Three sets of wild-type and null siblings were tested. One set ($n = 11$ for each genotype) was tested with denatonium benzoate and NaCl. The second set ($n = 12$ for each genotype) was tested with quinine sulphate and sucrose. The third set ($n = 12$ for each genotype) was tested with sucrose, HCl, and SC45647. Between tests, mice were provided with acidified water (pH 4.2–5.2) for ~2-week intervals. Tested mice ranged in age from 8–20 weeks. Mice were individually housed, provided with food *ad libitum* (Pico Lab Mouse Diet 20 no. 5058; PMI Seeds) and presented with distilled water in two sipper bottles for 48 h before testing. During each 48-h test period, a given concentration of tastant was provided in one sipper bottle and the other contained distilled water. After 24 h, volumes consumed were recorded, the bottles refilled, and positions reversed (to control for positional cues that might affect preference). Tastants were presented in ascending series. Preference ratios were calculated as the fraction of tastant consumed compared to total volume consumed. N values varied owing to accidental drainage of the sipper tubes when mice inserted bedding into sipper spouts. When this occurred, data for that concentration point were omitted. ANOVAs (strain $\times$ concentration) (Table 1) were performed on the data, excluding those animals not generating data at all concentrations. Mean preference ratios shown in graphs and independent-group t -tests were calculated from total collected data.

ablation of α -gustducin would block the coupling of bitter and/or sweet responsive receptors to the regulation of certain intracellular second messengers, thereby blocking bitter/sweet TRC responses such as depolarization and neurotransmitter release. Any bitter or sweet compound that acts primarily on an α -gustducin-coupled taste receptor would be expected to have diminished behavioural and electrophysiological effects in the absence of α -gustducin. Compounds that act primarily via other G proteins or that by-pass taste receptors to act directly on intracellular targets²⁰ would not be expected to be affected by α -gustducin deficiency. The absence of the α subunit of gustducin may also have significant secondary effects on TRCs, for instance by creating an excess of free $\beta\gamma$ gustducin subunits that may inhibit other G-protein α -subunits or directly regulate effector enzymes²¹. Presumably, sweet-responsive versus bitter-responsive TRCs containing gustducin are physically distinct cells. Alternatively, the same gustducin-positive TRC may respond to both sweet and bitter, but via different transduction pathways. Ultimately, direct electrophysiological recording from the gustducin lineage of

TABLE 1 Summary of analyses of variance (strain $\times$ concentration) on behavioural and neurophysiological data

Response		Preference*		Phasic response†		Tonic response‡	
Tastant	Effect	F value	P value	F value	P value	F value	P value
NaCl	S:	0.687	0.4171	0.060	0.8103	0.066	0.8003
	C:	134.017	0.0000§	79.623	0.0000	202.547	0.0000
	SC:	1.651	0.1871	1.850	0.1760	1.642	0.2117
HCl	S:	0.416	0.5271	0.498	0.4930	0.560	0.4677
	C:	118.852	0.0000	132.368	0.0000	27.505	0.0000
	SC:	1.040	0.3929	1.495	0.2429	0.080	0.9238
Denatonium	S:	68.614	0.0000	34.827	0.0000	15.754	0.0014
	C:	45.691	0.0000	80.897	0.0000	6.478	0.0011
	SC:	10.384	0.0000	7.954	0.0003	3.136	0.0353
QSO ₄	S:	32.204	0.0000	5.806	0.0304	7.045	0.0189
	C:	15.278	0.0000	114.061	0.0000	13.504	0.0001
	SC:	6.580	0.0000	5.486	0.0098	5.607	0.0090
Sucrose	S:	30.534	0.0001	14.569	0.0021	21.265	0.0005
	C:	21.520	0.0000	22.692	0.0000	20.775	0.0000
	SC:	10.293	0.0000	16.612	0.0000	17.653	0.0000
SC45647	S:	21.211	0.0002	42.066	0.0000	39.029	0.0000
	C:	20.294	0.0000	45.181	0.0000	20.422	0.0000
	SC:	8.707	0.0001	25.342	0.0000	23.294	0.0000

* S, strain; C, concentration; SC, strain $\times$ concentration. Based on data shown in Fig. 2.

† Based on data shown in Fig. 3.

‡ Based on data not shown.

§ P values < 0.00005 are presented as $P = 0.0000$.

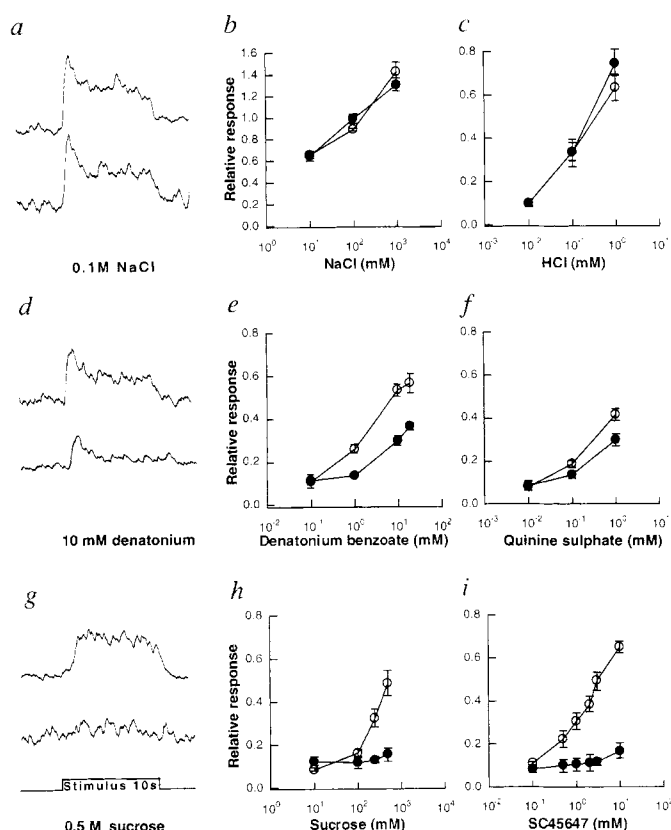


FIG. 3 Chorda tympani responses to lingual application of taste stimuli for wild-type control and α -gustducin null mice. *a, d, g*, Integrated neural responses of a wild-type (upper trace) and a null mouse (lower trace) to 0.1 M NaCl (*a*), 10 mM denatonium benzoate (*d*) and 0.5 M sucrose (*g*). *b, c, e, f, h, i*, Phasic responses of null ($n = 6$, filled circles) and wild-type mice ($n = 9-10$, open circles) to taste compounds. Data shown represent the maximum neural response amplitude occurring within 4 seconds of stimulus onset and are expressed relative to the response to 0.1 M NH_4Cl . Owing to the large response of the chorda tympani to NaCl, the y-axis of *b* ranges from 0 to 1.4; y-axes for the other tastants range from 0 to 0.8. *b*, Neural response to NaCl concentrations (0.01, 0.1, 1.0 M). No significant strain or interaction effects were present (P values > 0.05 ; Table 1). *c*, Neural response to HCl concentrations (0.01, 0.1, 1.0 mM). No significant strain or interaction effects were present (P values > 0.05 ; Table 1). *e*, Neural responses to denatonium benzoate concentrations (0.1, 1.0, 10.0, 20.0 mM). Results of t -tests are as follows: 1.0 mM ($P < 0.001$), 10.0 mM ($P < 0.0001$), 20.0 mM ($P < 0.01$). *f*, Neural response to quinine sulphate concentrations (0.01, 0.1, 1.0 mM). Results of t -tests are as follows: 0.1 mM ($P < 0.05$), 1.0 mM ($P < 0.01$). *h*, Neural response to sucrose concentrations (0.01, 0.1, 0.25, 0.5 M). Results of t -tests are as follows: 0.25 ($P < 0.01$), 0.5 M ($P < 0.001$). *i*, Neural response to SC45647 concentrations (0.1, 0.5, 1.0, 2.0, 3.0, 10.0 mM). Results of t -tests are as follows: 0.5 mM ($P < 0.05$); 1.0 mM ($P < 0.01$), 2.0 mM ($P < 0.001$), 3.0–10 mM ($P < 0.0001$).

METHODS. Male mice (Fig. 2 legend) were anaesthetized with sodium pentobarbital (60 mg kg^{-1}). Surgical, taste stimulation and recording procedures have been described³⁰. Neural signals were amplified ($10,000\times$) and integrated using an RMS-DC converter (Hendrick and Associates, Tallahassee, FL) with a time constant of 0.5 s. Taste solutions were presented for 10 s followed by a ≥ 1 -min rinse with distilled water at a constant flow rate of 3 ml min^{-1} . Excess fluid was removed through a suction tube positioned beneath the tip of the tongue. Each tastant concentration was presented at least twice and the mean response calculated. Tastants were presented as an ascending concentration series with presentation of 0.1 M NH_4Cl bracketing each concentration series. The mean phasic response to 0.1 M NH_4Cl was used to normalize responses within each bracketed concentration series. This concentration of NH_4Cl produced similar chorda tympani response magnitudes in null and wild-type mice. Data were analysed using strain $\times$ concentration ANOVAs for each tastant (see text and Table 1). *Post hoc* comparisons were performed using independent group t -tests (Fig. 3 legend).

TRCs from wild-type and mutant mice with α -gustducin point mutations may enable us to determine gustducin's specific roles in bitter and sweet transduction. □

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Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate

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CERAMIDE is an important regulatory participant of programmed cell death (apoptosis) induced by tumour-necrosis factor (TNF)- α and Fas ligand, members of the TNF superfamily^{1–6}. Conversely, sphingosine and sphingosine-1-phosphate, which are metabolites of ceramide, induce mitogenesis⁷ and have been implicated as second messengers in cellular proliferation induced by platelet-derived growth factor and serum^{8,9}. Here we report that sphingosine-1-phosphate prevents the appearance of the key features of apoptosis, namely intranucleosomal DNA fragmentation and morphological changes, which result from increased concentrations of ceramide. Furthermore, inhibition of ceramide-mediated apoptosis by activation of protein kinase C results from stimulation of sphingosine kinase and the concomitant increase in intracellular sphingosine-1-phosphate. Finally sphingosine-1-phosphate not only stimulates the extracellular signal-regulated kinase (ERK) pathway¹⁰, it counteracts the ceramide-induced activation of stress-activated protein kinase (SAPK/JNK). Thus, the balance between the intracellular levels of ceramide and sphingosine-1-phosphate and their regulatory effects on different family members of mitogen-activated protein kinases determines the fate of the cell.

Consistent with previous studies^{4,11}, increasing intracellular levels of ceramide in human promyelocytic HL-60 cells or U937 monoclonal leukaemia cells either by addition of the membrane-permeable ceramide analogue C₂-ceramide (Fig. 1a–d), or by treatment with sphingomyelinase (Fig. 1e–f), induced apoptosis. Exposure to sphingosine-1-phosphate (SPP) prevented

ceramide-induced apoptosis in both cell lines, as measured by oligonucleosomal DNA fragment electrophoresis (Fig. 1a, e) and a quantitative DNA fragmentation assay (Fig. 1f). SPP also markedly reduced genomic fragmentation and the morphological changes associated with apoptosis, as detected by *in situ* staining (Fig. 1b, d). The ability of SPP to prevent apoptosis induced by serum deprivation in HL-60 cells (Fig. 1a) may also be related to an attenuation of ceramide-mediated cell death, given that serum withdrawal results in marked accumulation of ceramide¹².

Because the binding of TNF- α and the Fas ligand to their receptors results in stimulation of sphingomyelinase, which catalyses the degradation of sphingomyelin to ceramide^{1–5,13–15}, we investigated the effects of SPP on apoptosis induced by these cytokines. TNF- α not only induced intranucleosomal DNA fragmentation in U937 cells (Fig. 2a), it also reduced survival, as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) dye-reduction assay (Fig. 2b), effects that were prevented by co-treatment with SPP. SPP also reduced TNF-induced DNA strand breaks and the expression of the apoptotic traits (Fig. 2c–f). In addition, SPP inhibited apoptosis induced by Fas ligation in Fas-expressing Jurkat T cells (Fig. 3c).

Activation of protein kinase C antagonizes apoptosis induced by TNF- α , Fas ligand, and ionizing radiation, suggesting that the diacylglycerol/protein kinase C pathway counteracts ceramide-mediated apoptosis^{4,11,15,16}. Moreover, inhibitors of protein kinase C induce apoptosis in haematopoietic and neoplastic cell lines¹⁷. Although the mechanism by which protein kinase C opposes ceramide-mediated apoptosis has not been determined, activation of this enzyme in diverse cell types stimulates sphingosine kinase activity, resulting in intracellular accumulation of SPP⁷. SPP prevented apoptosis induced by several inhibitors of protein kinase C, including the bisindolylmaleimide GF 109203X, H7, calphostin C, UCN-01 (7-hydroxystaurosporine), and chelerythrin, in Swiss 3T3 and U937 cells (Fig. 3a, b). To verify that the activation of sphingosine kinase by protein kinase C was responsible for inhibiting ceramide-mediated apoptosis, we used a competitive inhibitor of sphingosine kinase, N,N-dimethylsphingosine, which is a more potent inhibitor of sphingosine kinase than of protein kinase C¹⁸ (B.K. and S.S., unpublished results). At a concentration that markedly inhibited sphingosine kinase activity and formation induced apoptosis and prevented the ability of the protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), to suppress DNA fragmentation induced by Fas ligation in Jurkat T cells (Fig. 3c). SPP restored the cytoprotective activity of TPA and counteracted the DNA fragmentation induced by N,N-dimethylsphingosine (Fig. 3c). Results were similar with

TNF- α -induced apoptosis in U937 cells (data not shown). Activation of protein kinase C by TPA stimulated sphingosine kinase and increased SPP in both U937 cells (Fig. 4a) and Jurkat T cells (SPP increased from 11 ± 0.5 to 18 ± 0.9 pmol per 10^6 cells). Interestingly, the amount of SPP in cells exposed to TPA was similar to that in cells treated with a cytoprotective concentration ($1 \mu\text{M}$) of SPP (21.6 ± 1.3 pmol per 10^6 cells).

These results indicate that protein kinase C may be inhibiting ceramide-mediated apoptosis by activating sphingosine kinase, leading to increased cellular concentrations of SPP. TPA also increased sphingosine levels and, although it had no effect on the amount of basal ceramide, it blocked TNF- α -induced increases in ceramide (Fig. 4a). The latter effect was not mediated by activation of sphingosine kinase, because SPP had almost no effect on TNF- α -stimulated ceramide accumulation (Fig. 4a). Conversely, although TNF- α did not affect sphingosine levels, it reduced

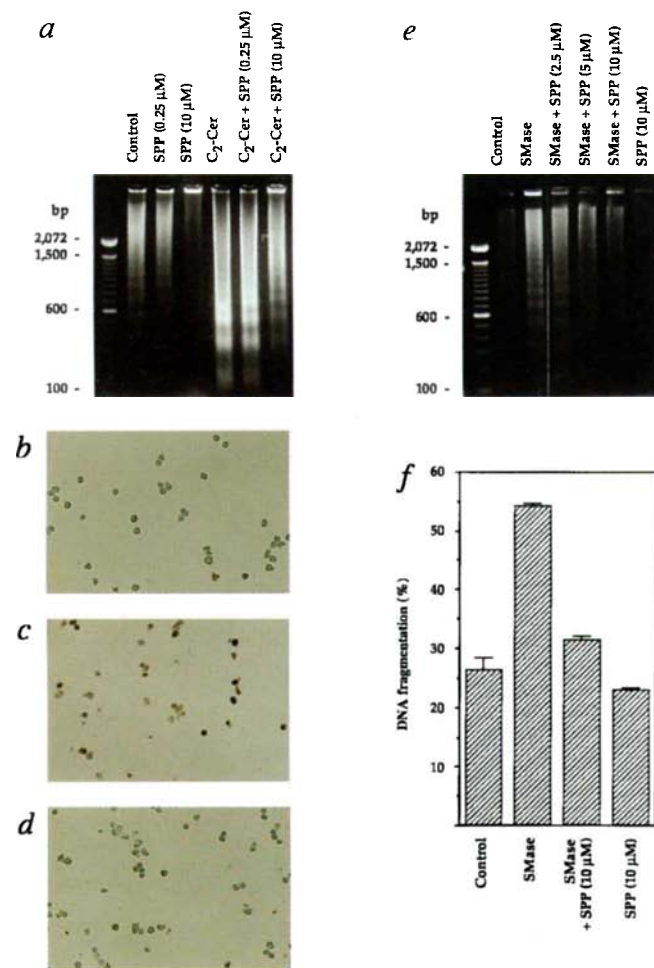


FIG. 1 Inhibition by SPP of apoptosis induced by *N*-acetyl sphingosine (C₂-ceramide) (a–d) or with sphingomyelinase (SMase), (e–f).

METHODS. Logarithmically growing HL-60 (ref. 11) (a–d, f) or U937 (ref. 4) (e) cells ($1 \times 10^6 \text{ ml}^{-1}$) were incubated in serum-free RPMI 1640 supplemented with insulin (5 mg l^{-1}) and transferrin (5 mg l^{-1}) and treated with vehicle or SPP (Biomol) added as a BSA complex²⁶, in the absence or presence of the indicated apoptotic agent. a, Cells were treated with $10 \mu\text{M}$ C₂-ceramide (Biomol) for 6 h, lysed, and their nucleic acids analysed by electrophoresis on a 1.8% agarose gel stained with ethidium bromide⁴. Left lane, 100-base-pair DNA ladder. b–d, Duplicate cultures were incubated without (b), or with $10 \mu\text{M}$ C₂-ceramide in the absence (c) or presence of $10 \mu\text{M}$ SPP (d), and apoptotic cells were detected by *in situ* staining with TACS-1 kit (Trevigen), which gives a dark brown insoluble precipitate indicative of genomic fragmentation. e, U937 cells were incubated in serum-free medium without or with 100 mU ml^{-1} *Staphylococcus aureus* sphingomyelinase (Sigma) in the presence of vehicle or SPP, and chromosomal-DNA fragmentation was assessed by agarose gel electrophoresis. f, HL-60 cells were incubated with [³H]thymidine ($1 \mu\text{Ci ml}^{-1}$) for 24 h to label DNA, and then washed before exposure to the indicated agents for 4 h. Cells were lysed and [³H]thymidine incorporated into both soluble and unfragmented DNA was determined by liquid scintillation counting²⁷; per cent fragmented DNA = $100 \times (\text{fragmented}/(\text{fragmented} + \text{intact chromatin}))$. Data are means $\pm$ s.d. All experiments were repeated 2–4 times with similar results.

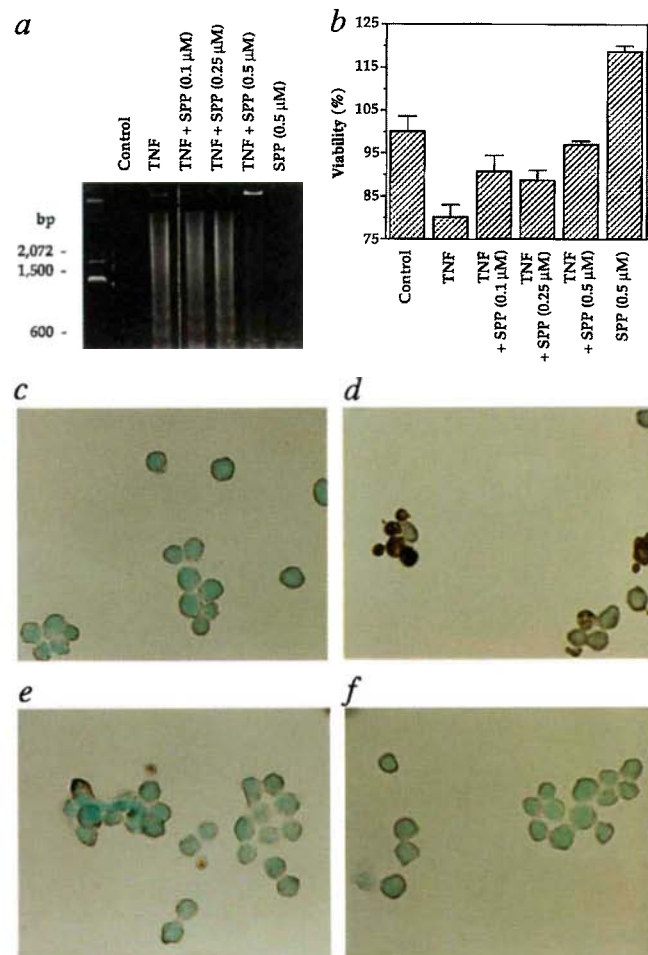


FIG. 2 Inhibition of TNF- α -induced apoptosis and enhancement of survival by SPP. a, U937 cells ($1 \times 10^6 \text{ ml}^{-1}$) were incubated in serum-free RPMI 1640 supplemented with insulin (5 mg l^{-1}) and transferrin (5 mg l^{-1}) and exposed for 3 h to TNF- α ($1,000 \text{ U ml}^{-1}$) in the presence of vehicle or SPP, as indicated. Cells were lysed and their DNA analysed by agarose-gel electrophoresis. Similar results were observed in three additional experiments. b, In duplicate cultures, U937 cell survival was measured using the MTT dye-reduction assay. After various treatments, cells were incubated with MTT for 4 h, solubilized with 10% SDS in 0.01 M HCl and cell viability assessed from spectrophotometry at 570 nm and 630 nm. The MTT dye reduction assay measures mitochondrial respiratory function and can detect the onset of cell death earlier than dye-exclusion methods. Viability was expressed as a percentage of that of untreated cells grown in serum-free medium. Data are means $\pm$ s.d. of triplicate determinations. c–f, Duplicate cultures were incubated as described without (c) or with $1,000 \text{ U ml}^{-1}$ TNF- α (d), TNF- α and $0.5 \mu\text{M}$ SPP (e), or $0.5 \mu\text{M}$ SPP (f). Apoptotic cells were then stained as for Fig. 1b–d. In different cell types undergoing apoptosis, the effective concentration of SPP might differ, depending on basal levels of ceramide, increases in ceramide induced by external stimuli, and the rate of degradation of SPP. High concentrations of SPP might induce apoptosis in some cell types by conversion to sphingosine and re-acylation to ceramide.

sphingosine kinase activity and markedly decreased the amount of intracellular SPP (Fig. 4a). Thus, TPA and TNF- α exert opposing effects on intracellular levels of both ceramide and SPP and therefore have a marked impact on the ratio of their concentrations. Although such a ceramide/SPP 'rheostat' may be characteristic of cell type, external stimuli can reset this ratio. Dissociation of growth-factor-induced mitogenesis from cytokine-induced apoptosis is a consequence of distinct sphingolipid-derived second messengers (Fig. 4c). In support of this idea, platelet-derived growth factor (PDGF) stimulates proliferation, in part, by activating ceramidase⁹ and sphingosine kinase⁸, increasing sphingosine and SPP^{8,9}, whereas inflammatory cytokines stimulate sphingomyelinase (but not ceramidase) activity, resulting in accumulation of ceramide⁹. Thus, regulation of sphingomyelinase, ceramidase and sphingosine kinase activities may determine the balance between cellular levels of ceramide that favour cell death and of SPP that inhibit death and is thus critical for the fate of the cell.

Paradoxically, it appears that the pathways of mitosis and apoptosis may be mechanistically related or tightly coupled. Growth factors such as PDGF stimulate the extracellular-signal-regulated kinases Erk1 and Erk2, whereas stress-activated protein kinases (SAPKs), also termed c-Jun amino-terminal kinases (JNKs)¹⁹, are activated by cytokines such as TNF- α ^{20,21}. In agreement with this, SPP stimulates the ERK signalling cascade in 3T3 fibroblasts¹⁰, whereas ceramide induces activation of JNK/SAPK in HepG2, HL-60 and U937 cells²⁰⁻²². As shown in Fig. 4b, TNF- α stimulated JNK activity, which peaked at 10–20 min and then declined, whereas TPA and SPP had no effect on JNK/SAPK activity yet stimulated ERK activity in U937 cells. Consistent with the central role of JNK/SAPK in ceramide-mediated apoptosis²²,

SPP also markedly inhibited the stimulation of JNK/SAPK activity by C₂-ceramide and TNF- α (Fig. 4b). In contrast, the ability of SPP to stimulate ERK signalling was not inhibited by TNF- α (Fig. 4b). Moreover, like SPP, TPA also antagonized TNF- α -induced JNK activity, whereas TNF- α potentiated the effect of TPA on ERK (Fig. 4b). These effects are consistent with the opposing actions of TNF- α and TPA on ceramide and SPP (Fig. 4a) and indicate that protein kinase C has multiple effects on the generation of sphingolipid metabolites and consequently on signalling. SPP stimulates ERK and inhibits JNK/SAPK; conversely, ceramide stimulates JNK/SAPK and inhibits ERK²¹.

Because the JNK/SAPK cascade is required for ceramide-induced apoptosis²², we investigated whether the ERK signalling pathway could also be important in the anti-apoptotic effect of SPP. Blocking the ERK pathway with PD 098059 (2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one; 10 μ M), a selective inhibitor of the ERK-activating kinase MEK²³, not only inhibited SPP-induced activation of ERK, it also prevented the cytoprotective effect of SPP on TNF- α -induced apoptosis in U937 cells. Furthermore, we found previously that Raf-1 is an important MEK activator for the response to SPP¹⁰. Thus, as a complementary approach we blocked ERK activation by transiently expressing a dominant-negative kinase-defective mutant of Raf-1 (K375W)²⁴. SPP no longer prevented TNF- α -induced apoptosis in U937 cells overexpressing this mutant (transfection efficiency >80%, based on β -galactosidase staining), whereas transfection with the control vector with no insert had no effects. Our results suggest that specific activation of different family members of mitogen-activated protein kinases are responsible for the opposing effects of ceramide and SPP on cell death and cell survival. It has been suggested that activation of JNK/SAPK with inhibition of ERK

FIG. 3 SPP reverses apoptosis induced by protein kinase C inhibitors (a, b) and opposes the effects of *N,N*-dimethylsphingosine, an inhibitor of sphingosine kinase, on apoptosis (c). a, Confluent quiescent Swiss 3T3 fibroblasts, cultured as described²⁶, were prelabelled with [³H]thymidine and treated in serum-free medium for 12 h with the indicated inhibitors of protein kinase C in the absence or presence of SPP (10 μ M), and DNA fragmentation determined as for Fig. 1f. Inhibitor concentrations were: UCN-01, 100 nM; H7, 5 μ M; GF 109203X, 20 μ M; calphostin C, 300 nM. b, U937 cells prelabelled with [³H]thymidine were treated for 5 h without or with H7 (10 μ M), calphostin C (150 nM), chelerythrin (8 μ M) or UCN-01 (25 nM) in the absence or presence of SPP (1 μ M), after which DNA fragmentation was determined. Data are expressed as per cent protection of DNA fragmentation compared to cells not treated with SPP. c, Jurkat T cells prelabelled with [³H]thymidine were treated for 12 h without or with TPA (25 nM), anti-Fas antibody (200 ng ml⁻¹), *N,N*-dimethylsphingosine (DMS, 10 μ M), or combinations as indicated, in the absence or presence of SPP (1 μ M) and DNA fragmentation was measured.

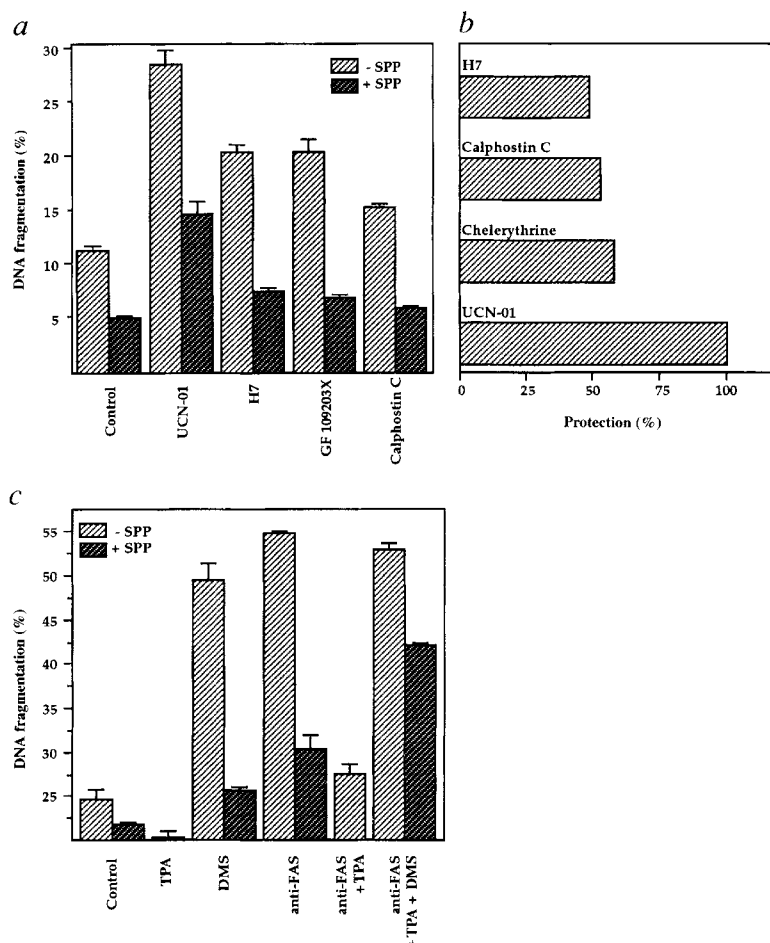
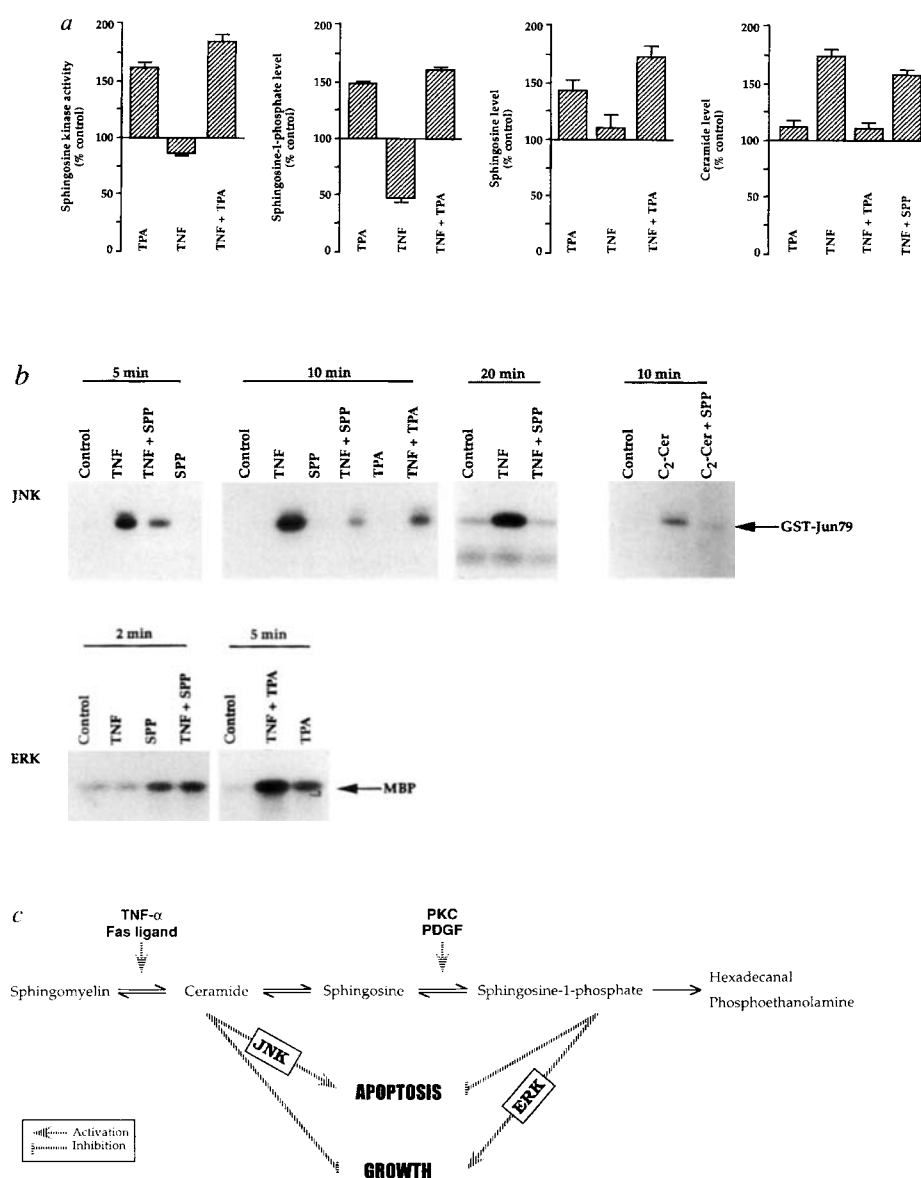


FIG. 4 *a*, Opposing effects of TNF- α and TPA on sphingolipid metabolites. U937 cells were treated with TPA (50 nM), TNF- α (1,000 U ml⁻¹), SPP (1 μ M) as indicated, lysed after 30 min and the activity of cytosolic sphingosine kinase determined⁸: basal activity was 17.3 $\pm$ 1.7 pmol min⁻¹ mg⁻¹. In duplicate cultures, lipids were extracted after 15 min and the amount of ceramide^{12,22}, sphingosine²⁸ and SPP²⁹ were measured; basal levels were 255 $\pm$ 13, 27 $\pm$ 3, and 9.6 $\pm$ 0.4 pmol per 10⁶ cells, respectively. Data expressed as percentage of values for control cells are means $\pm$ s.d. of triplicate determinations. *b*, Opposing effects of sphingolipid metabolites on ERK and JNK/SAPK signalling pathways. U937 cells were treated with TNF- α (1,000 U ml⁻¹) or C₂-ceramide (50 μ M) in the absence or presence of SPP (1 μ M) or TPA (50 nM). After various times, JNK activity was determined by retaining JNK with a recombinant GST-c-Jun79 fusion protein bound to glutathione-agarose beads and assessing its phosphorylating activity with the same fusion protein. JNK activity was also measured in an immune-complex kinase assay (20-min panel)³⁰. ERK was assayed with myelin basic protein as substrate after immunoprecipitation with antibody against p42^{MAPK} (ref. 30). Kinase reactions products were separated on 10% SDS-polyacrylamide gels. Data are representative of three similar experiments. *c*, Scheme depicting the effects of growth factors and cytokines on sphingolipid metabolism, leading to regulation of distinct members of the mitogen-activated protein kinase (MAPK) superfamily and culminating in cell growth or apoptosis. TNF- α and Fas ligand stimulate sphingomyelin hydrolysis to form ceramide, an activator of JNK/SAPK and an inhibitor of ERK, whereas PDGF or activation of PKC increases sphingosine kinase activity and SPP, a stimulator of the ERK signalling pathway of cell growth and inhibitor of the JNK/SAPK signalling pathway, leading to apoptosis.



could be critical for promoting cell death in rat pheochromocytoma PC-12 cells, whereas activation of ERK and suppression of JNK/SAPK promote cell survival²⁵. We propose that the dynamic balance between levels of sphingolipid metabolites, SPP and ceramide, and their effects on the ERK and JNK/SAPK pathways,

may determine whether a cell survives or dies. The beneficial effects of SPP in preventing apoptosis might be important in conditions such as AIDS, neurodegenerative disease, ischaemic stroke, and even normal ageing, in which programmed cell death is important. □

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A non-enzymatic p21 protein inhibitor of stress-activated protein kinases

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THE stress-activated protein kinases (SAPKs), which are identical to the c-Jun amino-terminal kinases (JNKs), are activated in response to a variety of cellular stresses, including DNA damage, heat shock or tumour-necrosis factor- α ^{1,2}. SAPK, a subfamily of the mitogen-activated protein (MAP) kinases, is a major protein kinase that phosphorylates c-Jun and other transcription factors²⁻⁵. SAPK phosphorylation of transcription factors is important in stress-activated signalling cascades¹⁻⁶. Here we report that the protein p21^{WAF1/CIP1/Sdi1}, a DNA-damage-inducible cell-cycle inhibitor⁷⁻¹⁰, acts as an inhibitor of the SAPK group of mammalian MAP kinases. This highlights a new biochemical activity of p21, which may provide the first evidence for a non-enzymatic inhibitory protein for SAPK. We suggest that p21, by inhibiting SAPK, may participate in regulating signalling cascades that are activated by cellular stresses such as DNA damage.

To investigate a possible role for p21 in regulating stress-activated protein kinase signal transduction, we examined the effect of recombinant p21 protein on the enzymatic activity of

SAPK- β (Fig. 1a). SAPK- β phosphorylation of a fusion protein substrate, glutathione-S-transferase (GST)-c-Jun, was stimulated after exposure of cells to ultraviolet light. Recombinant p21, as a form of GST fusion protein, potently inhibited SAPK- β *in vitro*. GST control did not affect SAPK- β activity. As SAPK/JNK includes several isoforms¹, we examined the inhibitory action of p21 on each subtype of SAPK. GST-p21 inhibited all subtypes of the SAPKs that we examined, including the α , β and γ isoforms (Fig. 1b). Interestingly, GST-p21 also inhibited an enzymatic activity of bacterially expressed p38 kinase (data not shown). p38 kinase, like SAPK, is another mammalian MAP kinase that can be activated by cellular stress¹¹. In contrast, extracellular-signal-regulated (ERK) MAP kinase was little affected, if at all, by GST-p21. GST-p21 used here is functionally active as a cyclin-dependent-kinase (CDK) inhibitor^{8,10} (Fig. 1b). GST-p21 inhibits both SAPK and CDK in a non-competitive inhibition mode (data not shown). The inhibitor constant (K_i) for SAPK- β or Cdk2 inhibition by p21 was 7.5×10^{-8} M or 1.7×10^{-8} M, respectively. Hexahistidine-tagged p21 also inhibited JNK1 activity in a manner similar to GST-p21 (Fig. 2a), suggesting that the GST portion of GST-p21 does not affect the inhibitory action of p21 on SAPK/JNK. To investigate the inhibition of SAPK/JNK by p21, we sought to identify the region of p21 that is involved in the inhibition of JNK1. The N-terminal domain (amino acids 1-84) of p21, p21(N), inhibited JNK1 activity, although this inhibition was less effective than by full-length p21 (Fig. 2a). In contrast, the carboxy-terminal half (amino acids 84-164) of p21, p21(C), had little inhibitory effect. Therefore, the inhibition by p21 of SAPK may be due, at least in part, to the N-terminal domain of p21. Interestingly, N-terminal domain of p21 inhibits CDK activity^{12,13}. Other small proteins can also act as CDK inhibitors¹⁴. To rule out

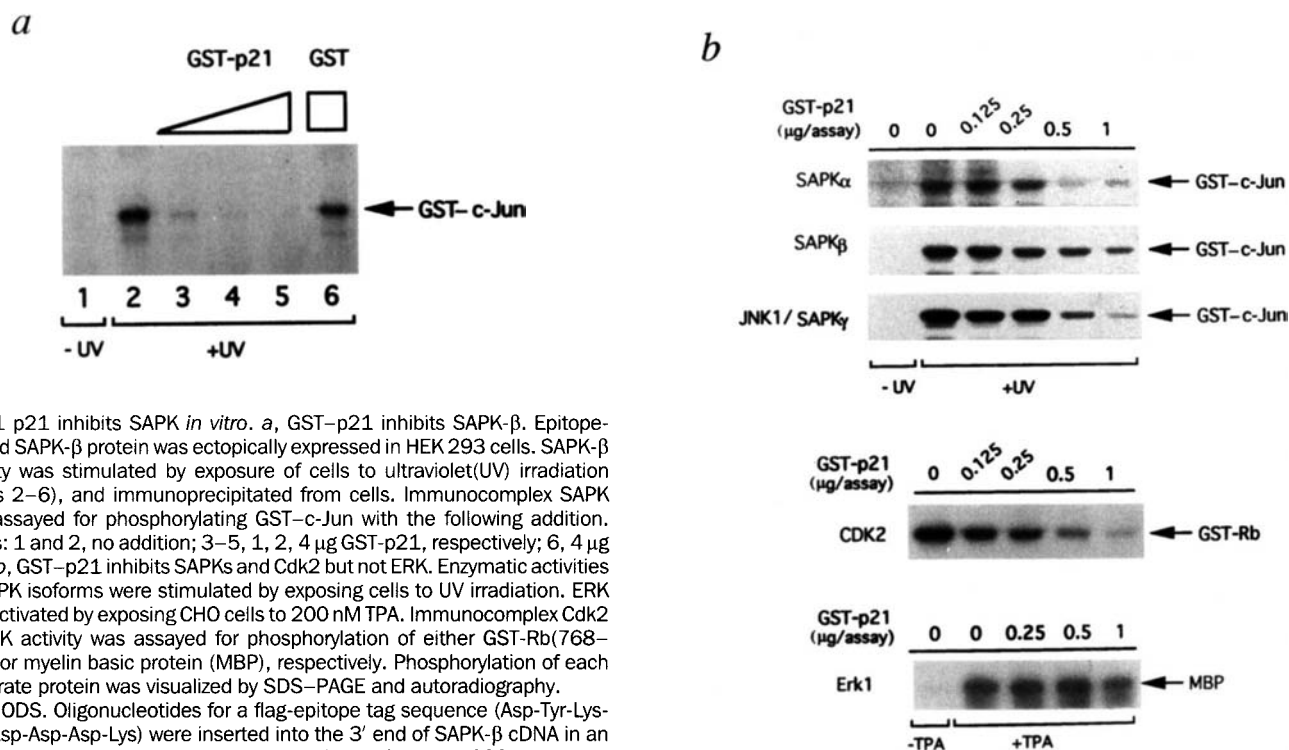


FIG. 1 p21 inhibits SAPK *in vitro*. **a**, GST-p21 inhibits SAPK- β . Epitope-tagged SAPK- β protein was ectopically expressed in HEK 293 cells. SAPK- β activity was stimulated by exposure of cells to ultraviolet (UV) irradiation (lanes 2-6), and immunoprecipitated from cells. Immunocomplex SAPK was assayed for phosphorylating GST-c-Jun with the following addition. Lanes: 1 and 2, no addition; 3-5, 1, 2, 4 μ g GST-p21, respectively; 6, 4 μ g GST. **b**, GST-p21 inhibits SAPKs and Cdk2 but not ERK. Enzymatic activities of SAPK isoforms were stimulated by exposing cells to UV irradiation. ERK was activated by exposing CHO cells to 200 nM TPA. Immunocomplex Cdk2 or ERK activity was assayed for phosphorylation of either GST-Rb(768-928) or myelin basic protein (MBP), respectively. Phosphorylation of each substrate protein was visualized by SDS-PAGE and autoradiography. METHODS. Oligonucleotides for a flag-epitope tag sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) were inserted into the 3' end of SAPK- β cDNA in an expression vector pcDNA1-neo (Invitrogen) by PCR. HEK 293 cells were stably transfected with plasmid expressing an epitope-tagged SAPK- β using the calcium phosphate method²⁹. Enzymatic activities of SAPK isoforms were activated by exposing cells to 40 J m⁻² UV-C and the cells lysed after 1 h. SAPK- α or JNK1/SAPK- γ was immunoprecipitated from HEK 293 cells with either anti-SAPK- α polyclonal antibody (UBI) or anti-JNK1/SAPK- γ monoclonal antibody (PharMingen), respectively. SAPK- β was immunoprecipitated from HEK 293 cells ectopically expressing SAPK β -flag with anti-flag M2 monoclonal antibody (Kodak). ERK activities were stimulated by exposing CHO cells to 200 nM TPA for 15 min. ERK was immunoprecipitated

from cells with anti-Erk1 polyclonal antibody (UBI) recognizing both Erk1 and Erk2. Cdk2 was immunoprecipitated from L929 cells with anti-Cdk2 polyclonal antibody (UBI). Immunocomplex kinase activity was assayed using 2 μ Ci [γ -³²P]ATP, 3 μ g substrate proteins and varying amounts of GST-p21, as described². Phosphorylation of the indicated substrate proteins was visualized by SDS-PAGE and autoradiography. GST fusion proteins were bacterially expressed using pGEX-2T (Pharmacia) and purified on glutathione-Sepharose.

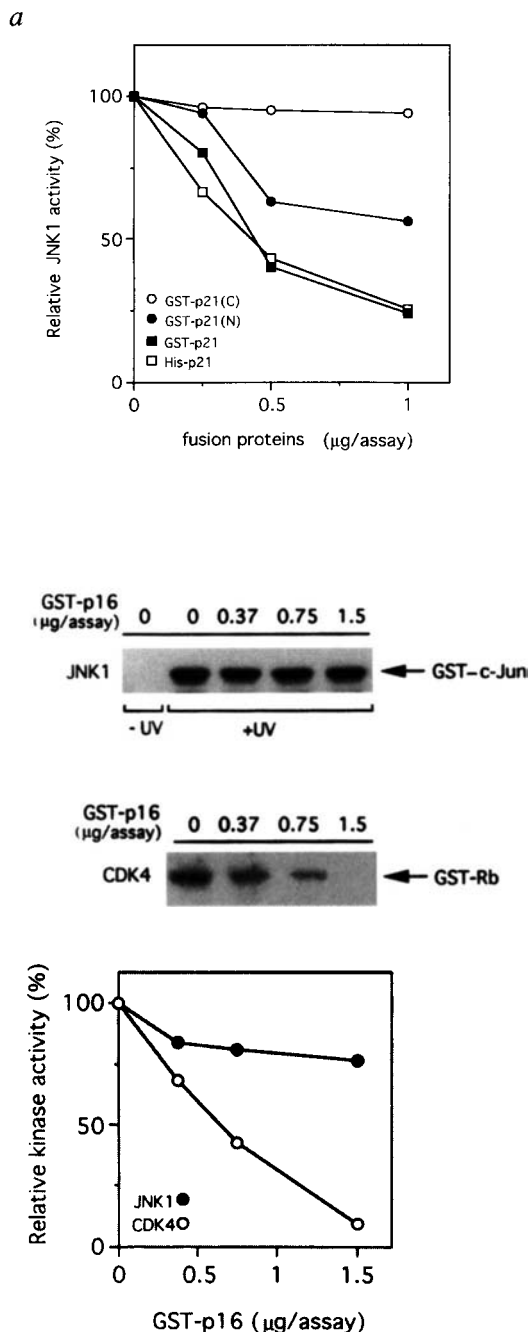


FIG. 2 SAPK/JNK is inhibited by p21, but not by p16^{INK4}. **a**, Effect of the N- or C-terminal region of p21 on JNK1 activity. UV-activated Jnk1 was assayed in the presence of full-length His-p21, full-length GST-p21, GST-p21(N) or GST-p21(C); p21(N), N-terminal domain of p21 (amino acids 1–84); p21(C), C-terminal domain of p21 (amino acids 84–164). **b**, GST-p16 inhibits Cdk4, but not Jnk1.

METHODS. Jnk1 activity was stimulated by exposing HEK 293 cells to 40 J m⁻² UV radiation and cells were collected after 1 h. Immunocomplex kinase assays for Jnk1 were done in the presence of the indicated amounts of fusion proteins, as for Fig. 1. Cdk4 was immunoprecipitated from 293 cells with anti-Cdk4 rabbit polyclonal antibody (UBI). Immunocomplex kinase assays for Cdk4 used GST-Rb(768–928) as substrate. Phosphorylated substrate proteins were subjected to SDS-PAGE, visualized by autoradiography and quantified by densitometry. Kinase activity was expressed as a percentage of that in the absence of fusion proteins. GST fusion proteins were bacterially expressed using pGEX-2T and purified with glutathione-Sepharose. His-p21 was bacterially expressed using pET-19b (Novagen) and purified with Ni²⁺-agarose.

the possibility that the inhibition of SAPK by p21 may be a general effect of CDK inhibitors, we examined the action of p16^{INK4} on Jnk1; p16^{INK4} is an inhibitor of the cyclin D-dependent kinases Cdk4 and Cdk6 (ref. 15) and has no structural homology with p21. We found that p16^{INK4} inhibited the catalytic activity of Cdk4 but not of Jnk1 (Fig. 2b).

We next tested whether p21 could bind SAPK/JNK. As shown in Fig. 3a, GST-p21 immobilized onto glutathione-Sepharose associates with *in vitro* translated [³⁵S]methionine-labelled Jnk1, but not with Erk1. GST-p21 also interacts with *in vitro* translated ³⁵S-labelled Cdk4. GST-p16 does not interact with *in vitro* translated ³⁵S-labelled Jnk1, although it binds strongly ³⁵S-labelled Cdk4 (Fig. 3a). Moreover, JNK1/SAPK-γ is detected by immunoprecipitation from *in vivo* ³⁵S-labelled proteins that bind to GST-p21, but not from *in vivo* ³⁵S-labelled proteins that bind to GST-p16 (Fig. 3b). The association of p21 with SAPK is also detected in a yeast two-hybrid *in vivo* assay¹⁶ (Fig. 3c). SAPK specifically interacts with p21 *in vivo*, but not with p16. p21 also interacts with Cdk2, but not with Erk2 in the two-hybrid assay (Fig. 3c).

To investigate whether the inhibition of SAPK by p21 occurs in intact cells, HEK 293 cells were cotransfected transiently with plasmid expressing SAPKβ-flag and pcDNA3 vector expressing p21 or pcDNA3 control vector (Fig. 4a), and SAPK activity was activated by ultraviolet irradiation of cells. This activation was markedly reduced in cells expressing recombinant p21 protein (Fig. 4a). Immunoblot data indicated that the inhibition of the SAPK activity by p21 did not result from a change in the level of expressed SAPKβ-flag. Many extracellular stimuli activate SAPK through Mkk4 (also named Sek1 or JNKK1), which, in turn, is activated by MEKK1^{17–19}; Mkk4 is a dual-specific threonine/tyrosine protein kinase that can phosphorylate and activate SAPK. We therefore examined the effect of p21 on the SAPK activity that was activated by the MEKK1/Mkk4/SAPK pathway in intact cells (Fig. 4b). Coexpression of both MEKK1 and Mkk4 proteins was able to activate SAPK. Expression of p21 resulted in a decrease in the MEKK1/Mkk4-activated SAPK activity. Expression of MEKK1, Mkk4 or p21 did not affect the levels of coexpressed SAPKβ-flag (Fig. 4b). In contrast, expression of p21 did not affect 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-activated ERK2 activity (Fig. 4c). Taken together, these findings demonstrate that cellular expression of p21 blocks activation of SAPK in cells. In addition, p21 prevents *in vitro* phosphorylation of SAPK by Mkk4 (data not shown), indicating that binding of p21 to SAPK may not only inhibit the catalytic activity of SAPK, but also prevent SAPK from being activated by Mkk4 in cells.

SAPK phosphorylates c-Jun and other transcription factors such as ATF-2 and TCF/Elk1^{2–5}. c-Jun interacts with Fos protein to form the AP-1 transcription factor²⁰, and with ATF-2 or other members of the ATF family^{3,4}. SAPK can stimulate AP-1 through phosphorylation of c-Jun⁶. Furthermore, SAPK phosphorylation of TCF/Elk1 may enhance c-Fos expression⁵. Thus, a major function of SAPK appears to be AP-1 activation⁵. Many types of cellular stress, including genotoxic stress, activate AP-1 (ref. 21). Upsetting the tightly regulated activity of AP-1 may cause cellular abnormality, as in oncogenic transformation, tumorigenicity or cell death^{22–24}. By modulating AP-1 and probably other transcription factors, SAPK mediates stress-activated signals in the cellular response to DNA damage or other stress. Furthermore, many cellular stresses that activate the SAPK pathway can also regulate p21 expression^{25,26}, so inhibition of SAPK by p21 may be an important mechanism for regulating stress-activated signalling.

p21 has two activities^{8,12,13,27}: one is to inhibit CDK, and the other to inhibit proliferating-cell nuclear-antigen-dependent DNA replication. Our results highlight a new function of p21, which is selectively to inhibit the SAPK group of MAP kinases. Interestingly, p21 does not inhibit the ERK group of MAP kinases. MAP kinase signal pathways are involved in a variety of biological events, including mitogenesis, differentiation and development²⁸. As each group of MAP kinases may mediate

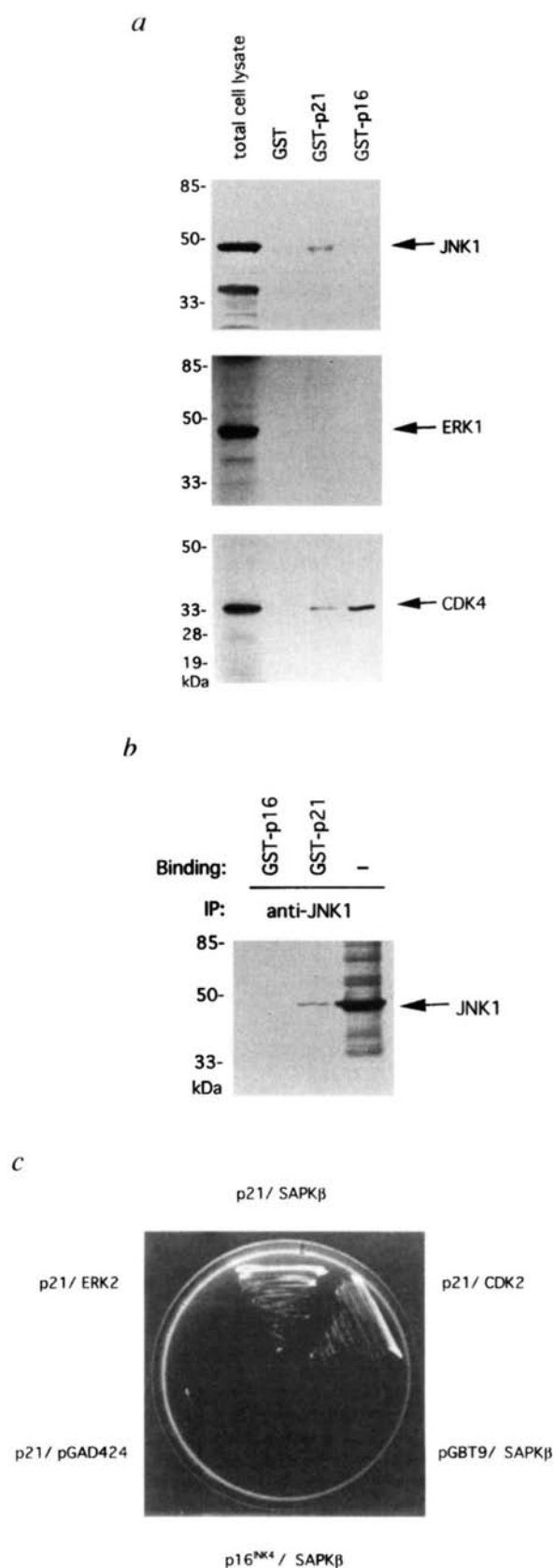
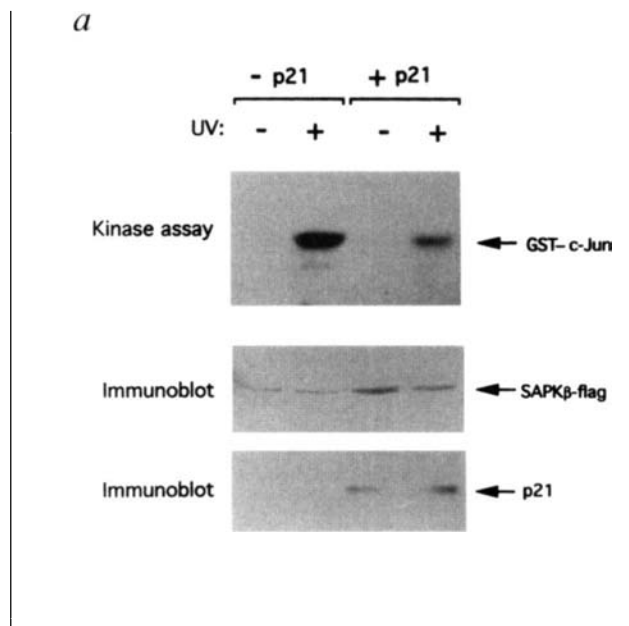


FIG. 3 p21 interacts directly with SAPK/JNK. **a**, Autoradiograms showing the binding of *in vitro* translated [35 S]methionine-labelled proteins to GST fusion proteins. Jnk1, Erk1 or Cdk4 was translated *in vitro* in the presence of [35 S]methionine. 35 S-labelled proteins were applied to GST, GST-p21 or GST-p16 immobilized onto glutathione-Sephadex. Bound proteins were washed extensively, eluted, and separated by SDS-PAGE. 35 S-labelled



proteins were visualized by autoradiography. **b**, *In vivo* 35 S-labelled JNK1/SAPKγ binds GST-p21. 293 cells were metabolically labelled with [35 S]methionine. *In vivo* 35 S-labelled cell lysates were directly immunoprecipitated with anti-Jnk1/SAPKγ monoclonal antibody, or were applied to either GST-p21 or GST-p16 immobilized onto glutathione-Sephadex. Resin-bound proteins were extensively washed, eluted, and immunoprecipitated with anti-Jnk1/SAPKγ monoclonal antibody. Immunoprecipitates (IP) were subjected to SDS-PAGE, and 35 S-labelled Jnk1 was visualized by autoradiography. **c**, p21 directly interacts with SAPK in a yeast two-hybrid system¹⁶. The genes encoding p21 or p16 were cloned into a GAL4 DNA-binding-domain vector, pGBT9. The SAPKβ, Cdk2 or Erk2 genes were cloned into a GAL4 activation-domain vector, pGAD424. Both pGBT9 and pGAD424, which encode two hybrid proteins, were cotransformed into the HF7c yeast host strain. Transformed colonies were replated on histidine-deficient medium to detect protein-protein interaction. When two hybrid proteins interact with one another, the HF7c yeast strain is expected to grow on histidine-deficient medium and express β-galactosidase. Colonies from the p21/SAPKβ, or p21/CDK2 cotransformants grew in the absence of histidine. Those colonies also turned blue on X-gal medium (data not shown).

METHODS. **a**, GST-p21, GST-p16 or GST control protein was immobilized onto glutathione-Sephadex. Jnk1, Erk1 and Cdk4 were *in vitro* translated by a rabbit reticulocyte lysate system (Promega) using [35 S]methionine. The translation product (18 μl) was incubated at 4 °C for 2 h with resin bound GST fusion proteins in 400 μl of binding buffer¹³. Beads were rinsed extensively with washing buffer (1 mM EDTA, 1 mM DTT, 0.1% Tween 20, 150 mM NaCl, 50 mM HEPES, pH 7.5). 35 S-labelled proteins were eluted from the resin and visualized by SDS-PAGE and autoradiography. **b**, Subconfluent 293 cells were starved for 30 min in methionine-deficient DMEM and metabolically labelled for 6 h with [35 S]methionine (100 μCi ml⁻¹). Cells were lysed in 50 mM HEPES, pH 7.5, containing 150 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF, 100 μM sodium orthovanadate, and 5 μg ml⁻¹ leupeptin, and microcentrifuged for 10 min. Soluble fraction of the lysates was precleared twice by incubation at 4 °C for 1 h with preimmune mouse IgG1, protein G-agarose, and glutathione-Sephadex. The precleared 35 S-labelled cell lysates (400 μl) were directly immunoprecipitated with anti-JNK/SAPKγ monoclonal antibody, or the same amount of 35 S-labelled cell lysates was applied to either resin-bound GST-p21 or GST-p16. Resin-bound proteins were washed and eluted as in **a**. Eluted 35 S-labelled proteins were immunoprecipitated with anti-Jnk1/SAPKγ monoclonal antibody. Immunoprecipitates were subjected to SDS-PAGE and 35 S-labelled JNK1/SAPKγ was visualized by autoradiography. **c**, HF7c yeast strain was cotransformed with pGBT9(p21)/pGAD424(SAPKβ), pGBT9(p21)/pGAD424(CDK2), pGBT9(p21)/pGAD424(ERK2), pGBT9(p21)/pGAD424, pGBT9/pGAD424(SAPKβ), or pGBT9(p16)/pGAD424(SAPKβ) plasmids, respectively. Colonies from cotransformants were replated on medium in the absence of histidine.

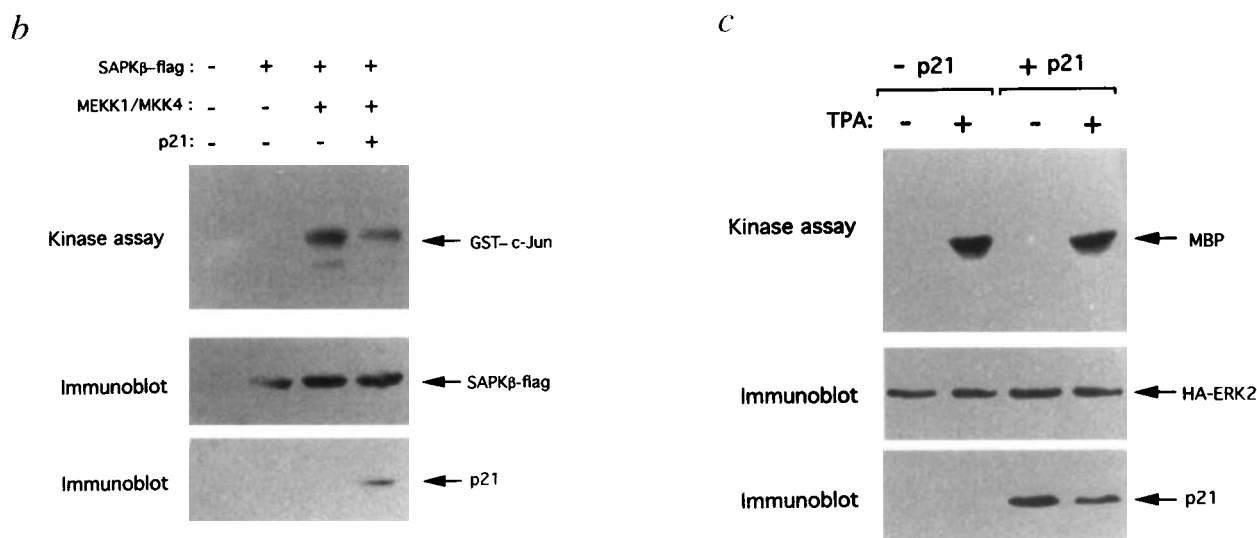


FIG. 4 p21 inhibits SAPK activity in intact cells. **a**, Coexpression of p21 results in a decrease in the UV-activated SAPKβ-flag activity. 293 cells were transfected with SAPKβ-flag or SAPKβ-flag and p21 as indicated. After exposing transfected cells to UV, cell lysates were assayed for SAPKβ-flag activity and immunoblotted for either SAPKβ-flag or p21. **b**, Effect of coexpressed p21 on the MEKK1/Mkk4-stimulated SAPKβ-flag activity. 293 cells were transfected with plasmids expressing indicated proteins. **c**, TPA-activated HA-Erk2 activity was not changed by coexpression of p21. **METHODS**. 293 cells were transiently transfected with pcDNA1-neo-SAPKβ-flag, pcDNA3-p21, pSRα-MEKK1, pcDNA3-MKK4, or pCEP4-HA-ERK2, as

indicated. For UV activation of SAPKβ-flag, transfected cells were exposed to UV (40 J m^{-2}) and the cells lysed after 1 h. For TPA activation of HA-Erk2, transfected cells were exposed to 200 nM TPA for 30 min. Immunocomplex kinase assays for SAPKβ-flag activity were performed as for Fig. 1. HA-Erk2 activity was isolated from cell lysates by immunoprecipitation with anti-HA monoclonal antibody (Boehringer Mannheim) and the immunocomplex kinase assayed using MBP as a substrate. Cell lysates were also immunoblotted with anti-flag, anti-HA or anti-p21 (Oncogene Science) monoclonal antibody using an enhanced chemiluminescence system (Amersham).

signal transduction for different cellular processes, p21 will be valuable for understanding the role of each MAP kinase subfamily in a network of complicated intracellular signalling cascades. Furthermore, our findings indicate that p21 may be a converging point for the regulation of cellular stress, cell cycle⁸, tumour suppression⁷, and senescence⁹. □

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Reduction of transcription by homologue asynapsis in *Drosophila* imaginal discs

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THE interactions between enhancers and promoter elements that control gene expression are generally considered to act in cis only, but genetic studies suggest that they can also function in trans between non-contiguous DNA molecules. Termed transvection¹, such trans interactions have been proposed to be responsible for several examples of intragenic complementation in *Drosophila*^{1–9}. Transvection is thought to depend on the physical proximity of sister chromosomes^{10,11}, because it is inhibited when chromosome rearrangements reduce the pairing of homologues^{1,3,5}. This led to the suggestion that transvection occurs when enhancer elements on one chromosome regulate expression on the other^{7,12}, with the pairing dependence resulting from a need for proximity between the two copies of the gene. Here we have analysed the levels of transcription from both alleles of the *Drosophila* *Ultra-bithorax* (*Ubx*) gene, and report that the predictions of this simple model are not supported. Our findings indicate a more complex level of trans regulation that may have implications for the aetiology of genetic disorders that are influenced by chromosome rearrangements.

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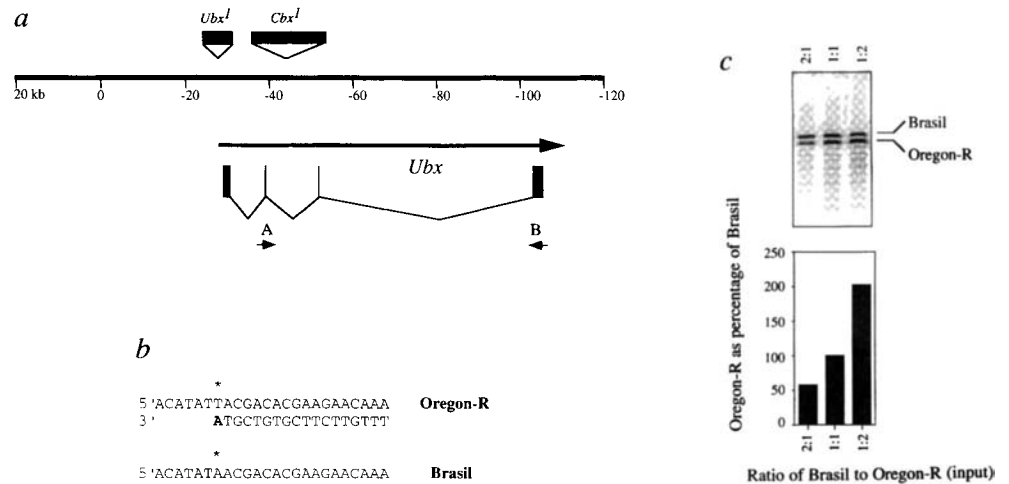
Ubx specifies haltere and abdominal identities in the *Drosophila* epidermis. Mutations that reduce its activity (for example null alleles such as *Ubx¹*, deletions of the locus, and some regulatory mutations; see Fig. 1a) transform these cells to wing¹³. In contrast, mutations that increase its activity (such as *Cbx¹*, which causes ectopic expression of *Ubx* in wing imaginal discs) transform wings to halteres. Unexpected interactions between *Ubx¹* and *Cbx¹* led to the initial observation of transvection. *Cbx¹ Ubx¹/+* heterozy-

gotes, in which the *Ubx* gene linked to the *Cbx¹* mutation is non-functional, nevertheless exhibit a transformation of wing to haltere. In such flies, the wild-type *Ubx* gene is presumably activated in *trans* by *Cbx¹*. Such transvection is suppressed by alleles of the *zeste* gene, for example *z^a*, and by chromosome rearrangements¹.

We developed a way to measure the distinct pools of transcripts produced by heterozygous alleles¹⁴, and used a variation of this

FIG. 1 a, Map of *Ubx* mutations. *Ubx¹* and *Cbx¹* are indicated above the solid line, which represents genomic DNA, and the large horizontal arrow indicates the primary transcript; numbers refer to kilobases. Arrows A and B represent the two PCR primers used to amplify *Ubx* cDNAs; A is in micro-exon 1 (nucleotides 1,762–1,782), B is in the 3' UTR (nucleotides 2,898–2,917). b, Portion of the *Ubx* 3' UTR sequence including the T → A polymorphism (asterisk at nucleotide 2,779) that distinguishes transcripts derived from the Brasil, R(B)3, R(B)4, R(B)8 and R(B)13 chromosomes from OR, *Cbx¹*, TM6b and 31316,607. Only two possible sequencing products, both terminated by ddA and differing from each other by a single nucleotide, can be made. The antisense sequencing primer shown below each template (2,797–2,819) was designed so that the first dT encountered by polymerase is at position 2,778 for Brasil and 2,779 for *Cbx¹*/OR cDNAs, providing unambiguous length distinction between sequencing products derived from Brasil (19 bp) or *Cbx¹* and OR (18 bp) templates. c, Allele-specific sequencing of *Ubx* cDNAs showing products derived from different ratios of OR and Brasil cDNA templates. cDNA prepared from either OR or Brasil haltere discs was prepared and amplified as described below, purified, quantified spectrophotometrically and mixed in the indicated ratios. For each sample, 1 ng was reamplified by PCR and processed as described below. The expected ratios (input) of OR to Brasil templates are plotted against experimental results (OR as per percentage of Brasil). Sequencing products of 18 bp (OR) and 19 bp (Brasil) are the only bands on the sequencing gel.

METHODS. Allele-specific amplification was performed as follows. cDNA was prepared from 1–3 mg imaginal discs as described¹⁴ using 50 ng *Ubx*



reverse transcriptase primer (GCAAACGGTTTGTGCGACTCC; nucleotides 3,051–3,072). A first strand cDNA sample (1 µl) was added to a PCR reaction (30 µl) with final concentrations of 0.4 mM of primers CGGCATATCAACAGACATGGG and TAGTCGTTTGGGCGAGAAC (nucleotides 1,762–1,782 and 2,898–2,917, respectively). Parameters for PCR were: 60 s at 94 °C, 60 s at 60 °C, 120 s at 72 °C for 36 cycles. The PCR product was purified by GeneClean following agarose gel electrophoresis and sequenced with primer AATATTCGATACTCCCGAAAACG (nucleotides 2,797–2,819) as described¹⁴ with the following modifications. Both template DNA and primer (~200 ng) were mixed in a total volume of 4.5 µl 10% DMSO. DNA was denatured at 94 °C for 1 min and frozen on dry ice. The combined labelling and termination reaction was performed in the presence of 1.5 mM dTTP, dCTP, ³⁵S dGTP (6,000 Ci mmol⁻¹) and ddATP. After electrophoretic separation, the peak height for each band was measured with a Phosphor-imager and background (typically less than 2% of signal) was subtracted.

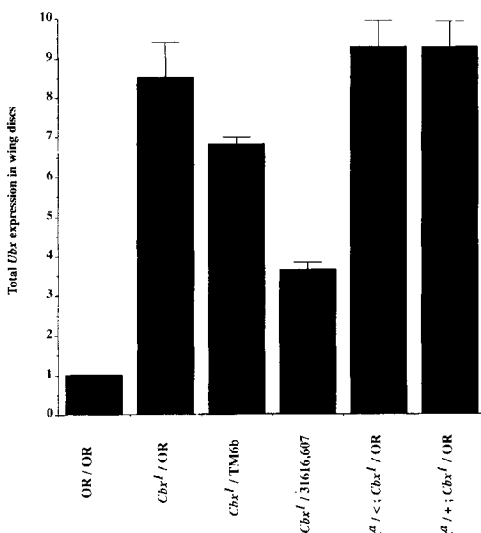
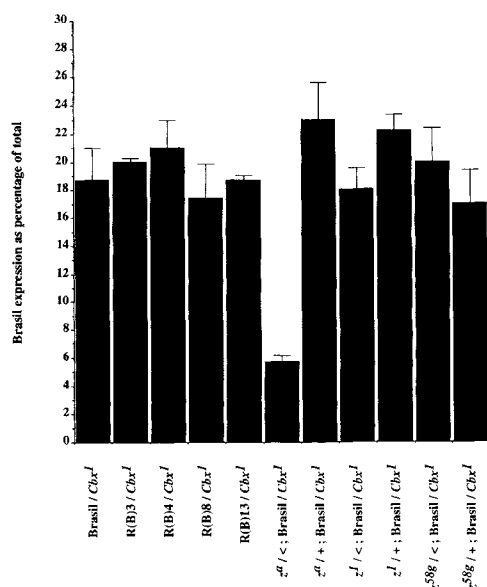


FIG. 2 *Ubx* transcription in *Cbx¹* wing imaginal discs is enhanced by chromosome synapsis. cDNA (2 µl) prepared from 4 or 8 similarly sized late third instar wing discs from the indicated genotypes was used for allele-specific amplification in the presence of 1 or 0.5 arbitrary unit of Brasil cDNA template prepared from haltere imaginal discs. PCR amplification produced different amounts of product from either the Brasil and non-Brasil templates, as indicated. At least three independent experiments were performed for each genotype.



method (Fig. 1b, c) to analyse transactivating interactions at *Ubx*. To quantify *Ubx* expression in wing and haltere imaginal discs, we took advantage of a single-base polymorphism in the *Ubx* 3' untranslated regions (UTRs) of Oregon R wild-type (OR) and Brasil wild-type flies. Complementary DNA was prepared from either a fixed number of OR or *Cbx^l*/OR imaginal discs, and these cDNAs were mixed separately with an arbitrary but fixed amount of cDNA standard prepared from Brasil discs. We observed that wing discs contain low levels of *Ubx* RNA, which is consistent with previous studies on *Ubx* protein¹⁵. Given that wing discs contain five times as many cells as halteres, we estimate that, on average, haltere cells express 43-fold more *Ubx* RNA per cell than do wing disc cells.

We then compared *Ubx* RNA in *Cbx^l*/OR and OR/OR wing discs, and examined the effects of chromosome rearrangements and *z^a*. Using Brasil cDNA for standardization, we determined that *Cbx^l*/OR wing discs had approximately 8.5-fold more *Ubx* RNA than did OR/OR wing discs (Fig. 2). This elevated level of *Ubx* RNA in *Cbx^l*/OR discs is consistent with the transformation of wing to haltere in these discs, and not unexpectedly the level fell in discs with transvection-suppressing chromosome rearrangements. Chromosome rearrangements that affected a wild-type (non-*Cbx^l*) chromosome, such as TM6b (which has multiple rearrangements that break sites distant from *Ubx*, and suppresses transvection moderately) and T(2;3)31616,607 (a strong suppressor of transvection), reduced total levels of *Ubx* RNA in a *Cbx^l* background by 20% and 58%, respectively. Thus chromosome rearrangements can reduce expression in a manner that directly correlates with the degree of chromosome asynapsis. In contrast, the level of *Ubx* RNA in wing discs did not change detectably in *z^a* mutants. We note that *z^a*; *Cbx^l*/+ flies do not differ significantly in phenotype from *Cbx^l*/+, whereas the wings of *z^a*; *Cbx^l* *Ubx^l*/++ flies are considerably less like halteres than those of *Cbx^l* *Ubx^l*/++ flies. We presume that the *z^a* mutation also reduces transactivation in *Cbx^l*/+ flies, but that it does not lower the total level of *Ubx* expression sufficiently for it to fall below a phenotypic threshold.

To assess the contributions of the two *Ubx* genes to the elevated levels of *Ubx* RNA in *Cbx^l*/+ wing discs, we assayed RNA extracted from *Cbx^l*/Brasil wing discs, and used the polymorphic sequence difference (Fig. 1b) in their *Ubx* 3' UTRs to distinguish their *Ubx* transcripts. Brasil transcripts contributed almost 20% of the total (Fig. 3), indicating that their abundance had been enhanced more than threefold compared to normal, and thus that *Cbx^l* had caused *trans*-activation. We then examined the identity of *Ubx* RNA in *Cbx^l*/Brasil heterozygotes that carried

FIG. 3 *Trans*-activation requires *z* function but not chromosome synapsis. RNA isolated from mature third instar wing imaginal discs of the indicated genotypes was subjected to allele-specific amplification (as Fig. 1). The sequence reaction products are expressed as a percentage of the total contributed by the Brasil template. R(B)3, R(B)4, R(B)8 and R(B)13 are Brasil strains with third chromosomes that have translocation and/or inversion breakpoints. These rearrangements suppress transvection. METHODS. Flies with rearrangement chromosomes were identified among the progeny of 150 Brasil males that had been irradiated with X-rays (4,000 rads) and then mated with *Cbx^l* *Ubx^l*/TM1 females. Flies with the indicated rearrangement chromosomes had weakened transformations of wing to haltere, and had rearrangement breaks proximal to 89E, the cytological location of *Ubx*. Transcript analysis was as Fig. 1.

various *z* alleles. *z^a*, but not *z^l* or *z^{58g}*, suppresses transvection¹⁶ and *z^a*, but not *z^l* or *z^{58g}*, reduced the transcripts from the Brasil *Ubx* gene to normal levels (Fig. 3). The amount of *Ubx* transcript transcribed from the *Cbx^l* gene was not altered in any of these mutants (Fig. 2). These data indicate that *z^a* suppresses transactivation of the wild-type *Ubx* gene by *Cbx^l*.

We next examined flies with chromosome rearrangements that alter homologue pairing at *Ubx*. We isolated flies with rearranged Brasil chromosomes in a screen for mutations that suppressed transvection in *Cbx^l* *Ubx^l*/++ flies. Among those selected in the screen, all had chromosome breaks in 3R, the chromosome arm containing *Ubx*. None of the chromosome breaks in these flies was in the cytological location of *Ubx*, 89E. These rearranged chromosomes (R(+)) suppressed transvection strongly (for example,

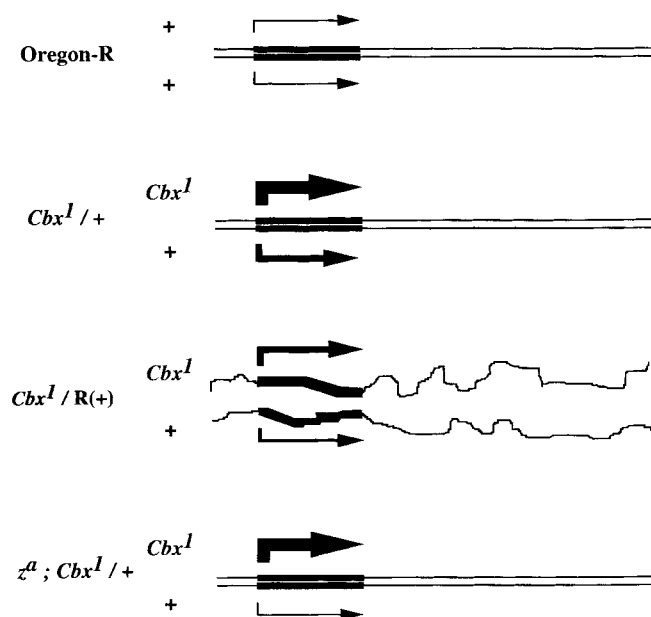


FIG. 4 Transcription of *Ubx* in wing imaginal discs. The quantitative relation between the amount of transcript contributed by each of the *Ubx* templates in the indicated heterozygotes is shown. Transcripts are symbolized by arrows, with their amount being indicated by the relative arrow size.

R(B)3; comparable to T(2;3)31616,607), moderately (for example, R(B)13), or weakly (for example, R(B)4, R(B)8), and they were used to construct *Cbx¹/R(+)* strains for analysis. In all of the R(+)/OR heterozygotes we studied, each of the *Ubx* genes produced half of the *Ubx* RNA in both wing and haltere discs (data not shown). As described above, the +*Ubx* gene produces approximately 20% of the *Ubx* RNA in *Cbx¹/+* wing discs, and transvection-suppressing rearrangements reduce the total level of *Ubx* RNA in wing discs (Fig. 2). Surprisingly, the proportion of *Ubx* RNA derived from the + chromosome did not change significantly in any of the R(+)/*Cbx¹* heterozygous wing discs (Fig. 3). This indicates that the rearrangements reduce *Ubx* expression from both homologues but do not eliminate trans-activation specifically.

Our results show that the *Cbx¹* mutation activates *Ubx* expression in the wing disc on both homologues, confirming the prediction that regulatory elements can function in *trans* (summarized in Fig. 4). Wing disc expression induced by *Cbx¹* was sensitive to *z* function and to chromosome asynapsis, observations that are consistent with previous proposals based on either phenotypic or molecular assays^{2,17,18}. However, we found an unexpected difference between the mechanisms through which the *z^a* mutation and chromosome rearrangements affect expression. Although chromosome rearrangements and *z^a* have essentially indistinguishable phenotypic consequences, namely, suppression of the *Cbx* phenotype, only *z^a* specifically disrupts *trans*-activation. Unexpectedly, chromosome rearrangements reduced expression from both homologues. These results suggest that, in normal wild-type flies, chromosome synapsis enhances expression of the *Ubx* genes on both homologues, implying that association between homologous chromosomes has a general enhancing effect on transcription. We presume that the systems in *Drosophila* in which transvection has been observed¹⁻⁹ are those in which gene expression levels can be measured with particular sensitivity. Transvection may therefore provide a useful means of studying the interactions between homologous chromosomes that can subtly influence gene expression. Our finding that interactions between homologous chromosomes seem to enhance expression levels from both chromosomes has many possible implications. In particular, we note that gross chromosome rearrangements, such as translocations, could give rise to global reductions in gene expression on the affected chromosomes. They could therefore contribute to disease states associated with haplo-insufficiency and cancer, and could modulate fitness during speciation. □

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CORRESPONDENCE and requests for materials should be addressed to T.B.K. (e-mail: tomk@cgl.ucsf.edu).

ERRATA

Non-chondritic platinum-group element ratios in the Earth's mantle

L. Pattou, J. P. Lorand & M. Gros

Nature **379**, 712–715 (1996)

THE date of submission of this Letter for publication was given incorrectly as 16 March 1994; this date should have been 25 September 1995. □

A neural basis for lexical retrieval

Hanna Damasio, Thomas J. Grabowski, Daniel Tranel, Richard D. Hichwa & Antonio R. Damasio

Nature **380**, 499–505 (1996)

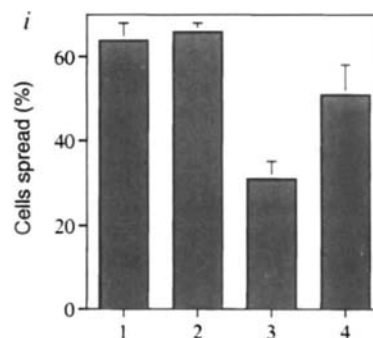
AN ambiguity was introduced during editing into the opening sentence of the heading of this Article. This should read “Two parallel studies, one conducted in neurological patients with brain lesions, the other using positron emission tomography in normal individuals, indicate that the normal process of retrieving words that denote concrete entities depends in part on multiple regions of the left cerebral hemisphere, located outside the classic language areas.” □

A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125^{FAK}

Alan Richardson & Thomas Parsons

Nature **380**, 538–540 (1996)

FIGURE 1, panel *i*, which was accidentally omitted from this Letter during the page-make-up process, is presented below. This figure shows the number of cells spread on fibronectin after 20 min. □



A new view on polarization microscopy

Rudolf Oldenbourg

Improvements to the traditional polarization microscope have enhanced the direct analysis of the molecular architecture in living cells with new electro-optical devices, polarization algorithms and digital image processing.

THE Pol-Scope was recently developed at the Marine Biological Laboratory in Woods Hole, Massachusetts, for the analysis of molecular architecture directly in living cells¹. Its capabilities were achieved by augmenting the traditional polarized light

microscope with novel electro-optical devices, algorithms to compute specimen birefringences and today's digital image capture and processing capabilities. Figures 1 and 2 exhibit the exquisite resolution, high contrast and analytical strength of images taken with this new microscope.

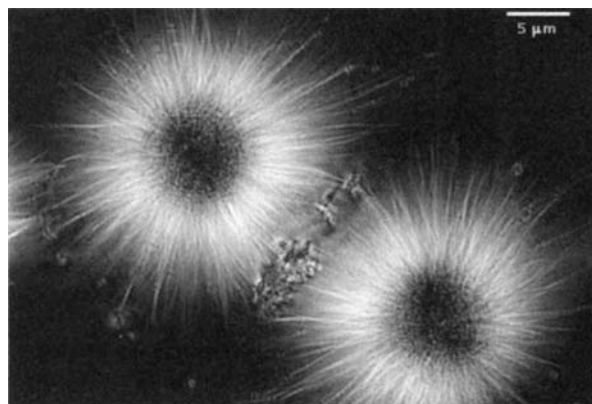


FIG. 1 Mitotic spindle isolated from fertilized sea urchin egg and imaged with the Pol-Scope (*Strongylocentrotus purpuratus*, preparation by John Murray, University of Pennsylvania, Philadelphia, USA). Retardance magnitude image, white: 5 nm retardance; black: 0 nm. Microtubule bundles radiate out from two black centrosomes; chromosomes visible between the two star formations (asters).

microscope with novel electro-optical devices, algorithms to compute specimen birefringences and today's digital image capture and processing capabilities. Figures 1 and 2 exhibit the exquisite resolution, high contrast and analytical strength of images taken with this new microscope.

The polarized light microscope has the capability to image and measure submicroscopic molecular order. The partial alignment of molecular bonds or of submicroscopic particles leads to birefringence, which alters the state of passing polarized light. Common examples include birefringent crystals, stretched plastic films and individual thin filaments and membranes, which can exhibit minute amounts of birefringence or dichroism. (Birefringence refers to the difference in refractive index for two orthogonally polarized light beams. Dichroism is the difference in absorption of those two light beams.) A careful analysis of the change in polarization of transmitted or reflected light reveals, and can be used to quantify, molecular order.

Based on its analytical power, the traditional polarized light microscope has found numerous applications in fields such as biology, mineralogy, metallography, chemistry and forensic studies²⁻⁵. In biology, the polarized light microscope has the potential to measure submicroscopic molecular order dynamically and non-destructively in samples that, in general, can be kept in native environmental conditions. Furthermore, specific structures, such as filaments and membranes, are highlighted owing to their intrinsic optical properties, without the need to stain or label them. With the traditional polarized light microscope, however, single images display only those anisotropic structures that have a limited range of orientations with respect to the polarization axes of the microscope. In addition, rapid measurements are restricted to a single image point or single area that exhibits uniform birefringence or other forms of optical anisotropy⁶, while measurements comparing several image points take an inordinately long time⁷.

To overcome the limitations of the traditional polarized light microscope, we incorporated a precision universal compensator made of two variable retarder plates¹. The optical setup (Fig. 3), in addition to a video camera and a specially designed, computerized image analysis system, provide fast measurements of specimen anisotropy (retardance magnitude and orientation; the word retardance was introduced by W. A. Shurcliff⁸ and refers to the relative change in phase between two orthogonally polarized beams traversing a birefringent specimen.

Expressed in distance, the retardance magnitude is the birefringence multiplied with the pathlength through the specimen. The slow axis orientation or retardance azimuth refers to the orientation of the principal axis with the largest refractive index). The retardance is measured at all points of the image and irrespective of orientation of the birefringence axes. Raw image data are recorded in less than half a second at a resolution of 640 by 480 image points and converted to retardance magnitude and orientation values in less than one second using a desktop computer. Retardance values as low as 0.02 nm and as high as several wavelengths can be measured, at a spatial resolution of 0.2 μm or larger, depending on the lenses used. Measurements are displayed as images representing submicroscopic molecular order at an unprecedented level of clarity and detail.

The optical design (Fig. 3) builds on the traditional polarized light microscope with two essential modifications: the specimen is illuminated with nearly circularly polarized light and the compensator is replaced by two electro-optical modulators (Fig. 4). The modulators are variable, linear retarders with their slow axes permanently adjusted at an angle of 45° to each other. While the axes are fixed, the magnitude of retardance can be changed by varying the voltages applied to the modulators. We have called this combination of two vari-

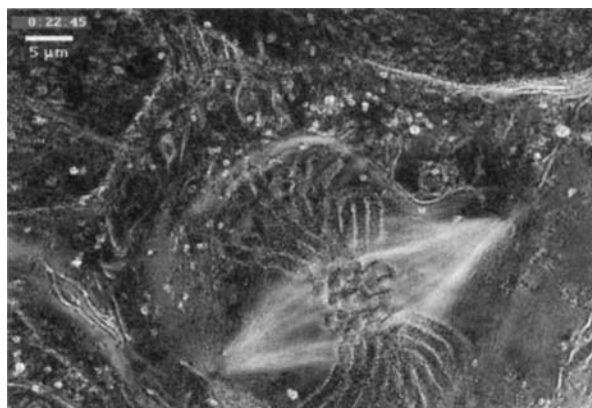


FIG. 2 Newt lung epithelial cell in mitosis (retardance magnitude image). Bright spindle fibres made up of microtubules locate the large chromosomes between the spindle poles. Chromosomes outlined by birefringence near their edges. (Collaboration with P. Tran and E. D. Salmon, University of North Carolina, Chapel Hill, North Carolina, USA.)

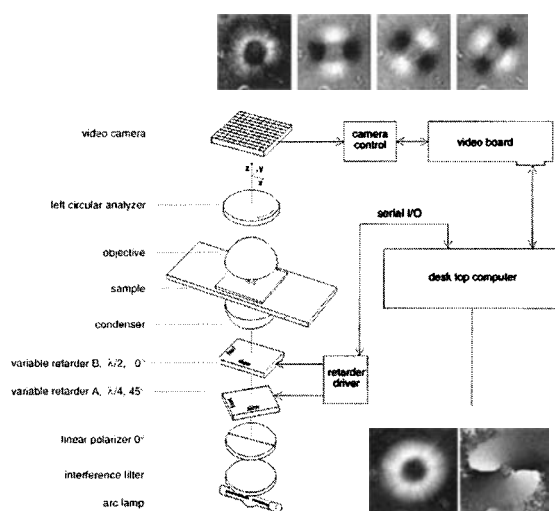


FIG 3. Schematic of the new Pol-Scope. The optical design (left) builds on the traditional polarized light microscope with the conventional compensator replaced by two variable retarders A and B forming the universal compensator. Images of the specimen (top row, aster isolated from surf clam egg) are captured at four predetermined retarder settings. The first image is in circularly polarized light (retarder A set to quarter wave and retarder B to half wave retardance). Subsequent images are in elliptically polarized light of different axis orientations. The monitor inset shows the computed retardance magnitude on the left image and on the right the azimuth image with brightness indicating slow axis orientation, from horizontal (black) to vertical (grey) to horizontal again (white).

able retarders a universal compensator for two reasons. First, it replaces the traditional compensator in the optical path of the polarizing microscope. Second, it can compensate any specimen birefringence, regardless of its orientation and magnitude, by changing the magnitude of retardances, that is the voltages applied to the



FIG. 4 The new universal compensator adapted to an upright microscope (Microphot SA, Nikon, Melville, New Jersey). Two liquid crystal (LC) variable retarders in rotatable mounts are located in the illumination path on top of the field lens near the base of the microscope stand. The electronic driver unit for the LC devices is sitting on the table to the left of the microscope stand. (LC devices and electronic driver unit from Cambridge Research & Instrumentation, Cambridge, Massachusetts, USA.)

modulators, so that a minimum of light equal to extinction passes through the analyser of the microscope.

To illustrate the workings of the universal compensator, four different images of the same birefringent specimen are shown in the top inserts of Fig. 3. The specimen is an aster (similar to one half of Fig. 1) formed in lysate prepared from eggs of the surf clam⁹. The aster is made from bundles of stiff filaments (microtubules) radiating from the centrosome in all directions. The bundles each possess a retardance of a fraction of a nanometre to several nanometres with the slow axis orientation parallel to the bundle axis. We emphasize that during the recording of all four images, no part of the microscope setup, including the specimen, was mechanically turned or moved. Instead, voltage were applied to the modulators were changed. Therefore, all four images recorded with the CCD camera are in perfect register, with corresponding picture elements representing intensities of the same object element.

Using the raw image data, the retardance magnitude and orientation images were computed. The algorithms were developed in our laboratory. They are based on a theoretical analysis of the polarization properties of the optical setup including the universal compensator, polarizers and the birefringence of the specimen. Recently, generally applicable algorithms have been developed by us for small and large specimen birefringences, measuring retardances of up to several wavelengths.

Many variations to the design shown in Fig. 3 are possible and already some have been implemented. For reflective samples (see Fig. 5), using an epi-illumination system, we have placed the universal compensator in the imaging path, after the half mirror, separating the illumination and imaging path in the epi-illumination micro-

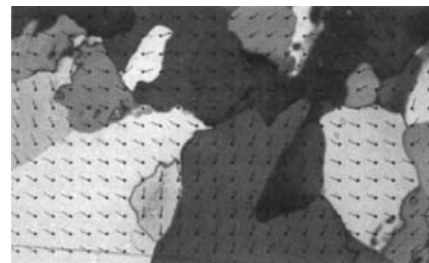


FIG. 5 Anodized aluminium surface observed in reflected light. Retardance magnitude shown as grey values, short lines indicate slow axis orientation. The retardance (up to 40 nm) is due to a thin layer (~1 nm thick) of birefringent alumina (Al_2O_3). The birefringence visualizes the grain structure at the surface of the metallic aluminium-lithium alloy. (Sample by Gary Laughlin, McCrone Research Institute, Chicago, Illinois, USA.)

scope. Moving the universal compensator from the illumination (as shown in Fig. 3) to the imaging side does not alter the algorithms significantly for computing retardance magnitude and orientation. In the future, we hope to show that the most general design of the Pol-Scope uses a linear polarizer and pair of linear retarders in both the illumination and the imaging path, sandwiching the sample between two universal compensators. We believe that this arrangement can measure all possible polarization optical parameters, including linear and circular birefringence and dichroism in the specimen, at the highest resolution of the light microscope.

With the approaching introduction of a commercial version of the new Pol-Scope (a commercial version of the instrument, called the LC-PolScope, is being developed by Cambridge Research and Instrumentation, Cambridge, Massachusetts, USA), we expect applications to develop in many different areas of pure and applied research. Because of its fast speed, high sensitivity, versatility and ease of use for measuring optical anisotropies, the new instrument significantly advances the analytical power of the polarizing microscope in all its traditional application areas. □

Rudolf Oldenbourg, Marine Biological Laboratory, Woods Hole, Massachusetts, USA. The author thanks Shinya Inoué of the Marine Biological Laboratory for his invaluable inspiration and support from the start of this development project. The instrument development is supported by the National Institutes of Health. For more information fill in reader enquiry number 100.

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Having a good look at microscopy

New equipment and technology for imaging and microscopy takes the stage in this product review, including a precision micromanipulation system, digital imaging and processing software, and a motorized arm for SEM.

PALM specializes in laser microscope systems for 'non-contact' **laser micromanipulation**. Designed for ease of handling, scientists and researchers should find the Optical Tweezers and ultraviolet-laser microbeam useful for various applications in basic research or patient treatment.



PALM's laser microscope system.

Recently, the company's Vis-OpticalTrap was introduced using a visible, high-quality diode laser system working at 680 nm. This wavelength is located within the so-called therapeutic window between 600 nm and 800 nm, where no water absorption and only weak absorption by biomolecules occurs. Therefore, trapping with 680 nm is less detrimental for living cells and organisms, as compared with the more common infrared trapping systems. The Vis-OpticalTrap can be focused to less than one micrometre in diameter and is available with a maximum power of 1,000 mW.

Reader Enquiry No. 101

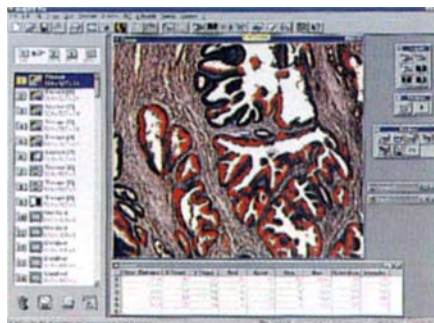
A new microscope for **precision micromanipulation work** has been introduced by Olympus Optical. The BX50WI has a fixed-stage, focusing nosepiece and infinity-corrected water immersion optics. It is designed for use with tissue or cell culture specimens and is particularly appropriate for neurophysiology or patch clamping applications, as well as intravital microscopy. The new slim-tip water immersion objectives provide improved image quality for the examination of tissue samples. They have working distances of 3.3 mm ($\times 10$, $\times 20$, $\times 40$) and 2.0 mm ($\times 60$), and respective numerical apertures of 0.3, 0.5, 0.8 and 0.9. The 43° insertion angle is the largest yet offered by Olympus, ensuring that there is sufficient clearance for head stages and monitoring electrodes. Good ultraviolet transmission ensures that the objectives are suitable for

measurement at 340 nm for fluorescence work, including observation of intracellular calcium. The BX50WI is suitable for fluorescence and differential interference contrast (DIC) observations. The DIC prism and components enable images to be obtained with high resolution and contrast, even from specimens as thick as 300 nm. A long working distance condenser designed for physiological applications adjusts for the thickness of various vessels. The stage is secured front and rear by a bridge support for extra stability. The low position of the stage allows an increased total focusing stroke, so that a broader range of vessels, including Petri dishes or perfusion chambers, can be used to house samples. Micromanipulators can be directly mounted on the stage support with adaptor brackets. The x/y stage movement makes it possible to bring a new portion of the specimen into view without disturbing the manipulators. Focusing is only through moving the nosepiece so that the microscope arm remains fixed to support accessories such as fluorescence and video components.

Reader Enquiry No. 102

Imaging tools

Synoptics offers a comprehensive range of **imaging software and hardware**, including high-performance colour and monochrome frame grabbers that are complemented by an extensive suite of Windows-based software for capturing,



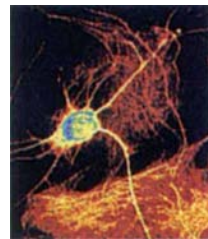
Synoptics' imaging software.

processing, analysing and archiving images. The latest PCI Prysm framestore allows the capture of high-resolution colour images from a wide range of camera inputs, including the latest digital cameras. For those wishing to develop their own imaging application software there is 'image objects' (IO), a new

approach to building imaging solutions for scientific and industrial applications. IO provides a range of imaging tools that make use of the new object linking and embedding technology (OLE) developed by Microsoft. Image archiving is designed to be simple by using the Treasury image database, which has been configured for microscopists to allow archives of micrographs to be readily linked to reports and analysis results from other Windows packages.

Reader Enquiry No. 103

Imaging Research offers **image analysis systems**, such as the microcomputer imaging device (MCID) and the analytical imaging station (AIS). MCID system's image-processing hardware support the full range of the company's software functions and use two discrete monitors to display images and data/control and interface to a broad variety of input devices. The AIS is designed to be more cost-effective and compact.



Imaging Research.

It does not include the specialized image processing hardware that is part of MCID and operates as a single-monitor system (image and data/control on one monitor). However, AIS contains many MCID functions for quantitative densitometry, morphometry, grain counting, counting, gels/blots, image display and enhancement applications. The 'turnkey' systems include everything needed to perform image analysis with the company offering setup and personnel training. Turnkey systems include Intel computers that are said to be sturdy and can be serviced worldwide. The 'system kit' MCID or AIS can be operated from the user's existing computer. MCID and AIS now use the OS/2 operating system, and will soon be available under the Windows 95 and Windows NT operating platforms.

Reader Enquiry No. 104

The AMBA 3.0 **image analysis system** from IBSB is an instrument for the processing and evaluation of images in industry and research. Through the integration of image recording and archiving, image processing and analysis, object recognition, classification and statistics, it should

assist researchers in technology, biology and medicine in complex imaging problems. In the system, there are numerous standard algorithms that can be implemented for image processing and image analysis, including image filtering and image transformation, DFT and FFT, binary-image segmentation, contour following and optimized grey-scale image segmentation, as well as histometry on the basis of Voronoi diagram and Delaunay graph, watershed generation, skeletonization and line analysis. It contains basic modules for image processing: colour space transformation, colour reduction and colour image segmentation. The integrated program environment includes an editor, a compiler and a debugger for efficient program development. It is possible for simple line-oriented programs to be written in DOS, as well as in the Windows environment. Applications of this system include semi-automated karyotyping, analysis of muscle and nerve morphometry, AgNOR analysis, DNA photocytochemistry, karyometry/histometry, latex spheres and material analysis.

Reader Enquiry No. 105

A new brochure from Edax covers **digital imaging and processing software** for speed and flexibility in X-ray microanalysis. The brochure details the new iDXprime digital imaging and processing software, which is designed to bring speed, functionality and

flexibility to X-ray microanalysis. It covers the features and benefits of the program, which operates on the Windows platform. Foremost among these is that it allows for the straightforward collection of images and X-ray maps, without the clutter of multiple open windows. It should prove useful for spectra collection, editing, exporting and saving of data in a single package, as well as for electron image and X-ray map acquisition. The brochure further details the image overlays and frame operations, median and selected kernel filtering, and image enhancement through histogram manipulation. Other topics of interest include light element mapping, image preview and galleries, high-resolution X-ray mapping, area fraction measurement, DX-Qmap and/or matrix effects, DX-live for acquiring X-ray images in seconds without compromising resolution and iDXline for creating high-quality, digital X-ray line scans.

Reader Enquiry No. 106

Scanning and probing

The LEO 982 is an ultra-high performance **field-emission SEM** that includes Gemini electron optics. This system is fully computer-controlled with advanced image processing, storage and archiving, as well as networking compatibilities. The

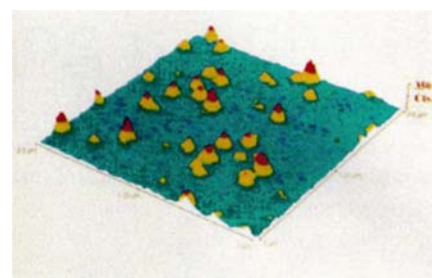


The LEO 982 field-emission SEM.

newly developed analytical sample chamber accommodates even the largest samples and allows simultaneous attachment of many signal detectors and accessories. It should provide SEM with enhanced image resolution throughout the entire beam-energy range, particularly at low-beam energies down to 200 eV. The analytical capacities have also been improved by using new electron optics, increased stability, high resistance to stray magnetic fields and floor vibrations, and versatile sample handling under high-resolution or analytical operating conditions.

Reader Enquiry No. 107

TopoMetrix offers single-molecule detection (SMD) using its Aurora **near-field**



TopoMetrix — single molecule detection.

scanning optical microscope (NSOM). This technology offers many of the capabilities of optical microscopy, as well as the high resolution (< 50 nm) of a scanning probe microscope (SPM). It has also been demonstrated that SMD is possible on the Aurora system using NSOM and Dil dye. The experiment involves spreading a submonolayer of dye molecules on a surface from a dilute solution so that single molecules may be isolated under the NSOM probe. Evidence that SMD has been achieved comes from the knowledge of the sample information. The ability to do SMD may provide a benchmark for the high sensitivity of the NSOM instrument. It may permit the acquisition of more specific information about the chromophore because the detected signal is no longer an average of the signal collected from multiple molecules. This should provide the freedom to minimize the degree of external tagging of biological specimens for fluorescence imaging. The Aurora can be modified to accommodate the use of avalanche photon counting and an oil-immersion transmission objective lenses.

Reader Enquiry No. 108

A **multifunctional probe** that resolves the problems of near-field, super-resolution optical imaging, while fully integrating such imaging into conventional scanned probe microscopes, has been introduced by Nanonics. The aim of these near-field optical elements is to allow subwavelength points of light to be brought within the near-field of a surface that is to be interrogated in conventional scanning probe microscopes. This probe utilizes a cantilevered structure in which the fibre tip is bent and coated so that light can be transmitted to the tip with the same efficiencies as those of conventional, straight, tapered near-field optical elements. According to the manufacturer, these cantilevered probes have all of the force characteristics of the silicon atomic force sensors, with resonance frequencies



Nanonics probe.

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up to 380 kHz and force constants down to 0.1 N m^{-1} . However, they also have near-field optical capabilities. The bent optical fibre readily allows light to be brought into the near field of a sample in any scanning probe microscope, thus broadening the use of near-field optics.

Reader Enquiry No. 109

A new three-position **motorized arm for use with scanning electron microscopes (SEMs)** has recently been released by K E Development. This facility allows the backscattered detector to be positioned automatically under the final lens for imaging and retracted when not in use. The motor drive is designed for detector assemblies that are mounted in inconvenient places, such as behind the electron-optical column, or where the whole chamber is positioned in a hazardous environment such as 'hot cells'. The motorized arm is configured to accommodate both the backscattered detector and a Faraday cup, which may be connected to a meter for accurate measurement of the probe current. This allows operating conditions to be accurately reproduced from sample to sample and is also important if quantitative X-ray analysis is also being performed. The motorized arm has a maximum retraction distance of 125 mm, with a positional accuracy for both backscattered and Faraday cup positions of $50 \mu\text{m}$. In addition to the motorized movement in the y-axis, a provision is also made for manual adjustments of $\pm 1 \text{ mm}$ in the x-axis and $\pm 10 \text{ mm}$ vertically. The system is equipped with a sensitive touch sensor that will stop movement of the arm immediately if it comes into contact with any electrically conducting object.

Reader Enquiry No. 110

The Zeiss LSM 310 **confocal laser-scan microscope** utilizes failure analysis techniques, which include high-resolution confocal imaging, emission microscopy, OBIC and infrared imaging. Recent additions to this system include techniques for fluorescence microthermography (FMI), backside emission, backside OBIC, LIVA and liquid crystal. The latest addition to this workstation is the introduction of several wafer probing options. Manual probing, as well as the use of probe cards, is now possible on the LSM. Its stand, stage and focus mechanism have been redesigned to provide a large working area under the microscope objective (12–23 cm). This increased space provides the flexibility to work with large test boards, a probe platen or a probe card holder. It is said to be easy to switch from one setup to another. A new probe card holder has been developed by Atto Instruments, which is designed to eliminate the need for x, y, z movements of the microscope. This helps to maintain the stability of the microscope, which is critical

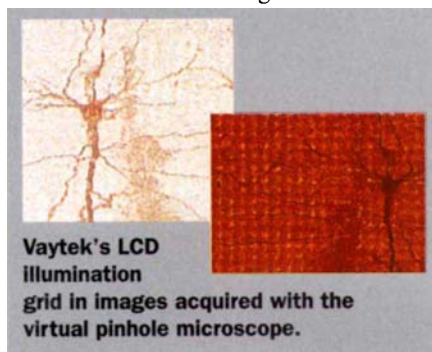
for the high magnification (greater than $\times 10,000$) work of the LSM.

Reader Enquiry No. 111

Polysciences now offers Moxtek's new **magnification reference standards for scanning electron microscopy (SEM)** with either one-dimensional parallel ribs or a two-dimensional grid pattern for easier imaging. Each individual standard is an original produced directly from a holographic interface pattern, yielding better accuracy, repeatability and uniformity. Two nominal calibrated dimensions are available. A data sheet provided with the standard will provide the actual dimension to three significant figures. The 700 nm standard provides accurate multiple period measurement from $\times 5,000$ to $\times 45,000$; the 300 nm standard is accurate from $\times 10,000$ to over $\times 100,000$. These standards are available as unmounted $3 \text{ mm} \times 4 \text{ mm}$ pieces to be mounted on the appropriate SEM stub.

Reader Enquiry No. 112

VayTek recently announced the development of a prototype for the 'scanning computed confocal imager' or **virtual pinhole microscope**. This technology takes advantage of existing improvements in digital imaging systems but configures functional subsystems in a way that is designed to eliminate many of the limitations and much of the expense associated with conventional confocal imagery. One of the key elements of the new imager is the use of a



'virtual' aperture, which is implemented in software on a host computer, such as a pentium-based computer or a Power Mac. It is possible to adjust key parameters, such as effective aperture size, to optimize the image even after the basic data have been acquired. Several algorithms are used to produce images similar to those acquired through conventional confocal microscopy. A key subsystem of the virtual pinhole microscope provides scanned illumination. Users can use spatial light modulators (SLMs) to produce time-variable, spatial patterns of illumination. The system uses liquid crystal displays (LCDs) similar to those used in some video projectors. By setting the polarization on the LCDs, it is possible to programme light patterns from a single point to a complicated spatial pattern (one or more lines or slits, choosing

optimal patterns in space and time for capturing the desired images).

Reader Enquiry No. 113

Philips Electron Optics has joined up with ElectroScan to produce ESEM, a new type of **scanning electron microscope (SEM)**. It is designed for use under 'wet' conditions, such as those frequently encountered in environmental research. The instrument combines the high brightness of a field-emission electron gun (FEG) column with the ESEM's ability to extend secondary imaging well into the 600 Pa vacuum range (where hydrated samples can be stable). By preventing dehydration of wet specimens, the new instrument should be well suited to observing samples in their natural environment, while reducing or eliminating the need for sample preparation. High-magnification, noise-free images can be obtained, even from materials containing elements with low atomic mass (low Z), as there is no charging of uncoated, non-conductive specimens. While the instrument normally uses water vapour as a convenient chamber environment, the operator can easily select an external source of gas (for example, nitrogen or air). Optional extras include the Everhart-Thornley detector for use in high-vacuum modes, solid-state and scintillator backscatter detectors, a specimen current detector and an integrated EDX detector.

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Accessories

For users looking for increased speed, flexibility and reliability in PC-based imaging systems, Data Translation has implemented a third-party software program that promotes **compatibility between the Mach series of real-time, PCI frame grabbers and third-party software applications**. Several image processing and machine vision applications, from vendors such as Integral Vision, Media Cybernetics and Western Vision Software, now support the Mach Series under Windows NT, Windows 95 and Windows 3.1. The Mach series of frame grabbers (DT3155 and DT3152 for PCs, and DT3155-PM for the Power Macintosh) perform accurate, real-time image capture and transfer by using the fast 32-bit PCI bus. Operating as PCI bus masters, the frame grabbers transfer image data continuously to the system display or memory, up to the amount of memory in the computer, with negligible CPU intervention. Each board accepts four monochrome inputs, including RS-170/NTSC or CCIR/PAL, and effectively provides eight bits of greyscale resolution.

Reader Enquiry No. 117



Data Translation's PC-based imaging systems.

Unlike many conventional photomicroscopy systems, the H-III **automatic camera system** from Nikon uses a swing-out beam splitter to ensure that 100 per cent of the available light reaches the film, offering microscopists improved clarity and quality. Film speeds, as low as ISO 6 can be used with this system, providing fine-grain photographs and sharper images. Even when there is little light available, internal reflections are reduced by a direct projection system. With this system, the image formed by the objective lens is projected directly on the film, rather than being relayed by a second lens. With fewer lenses, there are fewer reflecting surfaces and a sharper, flare-free image is possible. A 'V' groove mounting has been added to provide extra stability, as well as to reinforce the connection of the microflex to the phototube, enhancing image quality when long exposures are required. Photography is triggered using a simple cable release mechanism. An LED display ensures that all settings and readings can be easily seen. One per cent spot and 35 per cent average exposures, ranging from 0.01–999 seconds, are possible using the automatic settings. Film speeds ranging from ISO 6–20,000 can be used. Projection lenses

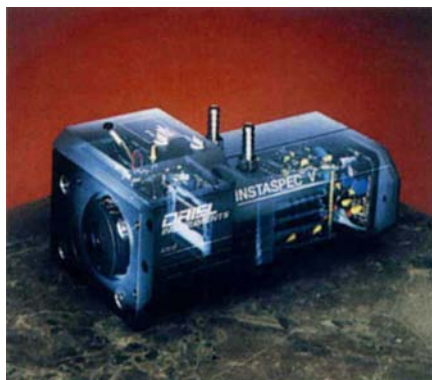
are available in $\times 2$ (large format only), $\times 2.5$, $\times 4$ and $\times 5$ magnifications.

Reader Enquiry No. 115

Axon Imaging Workbench 2 (AIW) is an effective, practical and easy-to-use **program for ion and analyte concentration imaging**. AIW runs under Windows and Windows 95. It is specifically designed for imaging biological preparations in low-light conditions, and for control of experiments in which ratiometric and non-ratiometric methods are used to determine ion concentration information as a function of time. The program can also perform on-line analysis (for example, during the experiment), with continuous update of graphs and data export, post-experimental analysis of imaging data, and synchronization of imaging with electrophysiology hardware and software. AIW can acquire, perform and display up to five or more full ratio images per second, even when using an inexpensive PCI bus frame grabber; still higher rates are said to be possible with the company's lightning board. It is said to have 'fumble-free' experimental control, using a handy tool bar that provides improved control in darkened rooms without having complicated dialogue boxes nested many levels deep. The experimental protocol can be done in the dialogue boxes, and once the experiment starts, the tool bar can be used for most operations.

Reader Enquiry No. 116

LOT-Oriel has announced the addition of **intensified CCD (ICCD) systems** to their multichannel product line. The high sensitivity and detectability of the new ICCDs make them well suited for Raman, luminescence, microspectroscopy and imaging. The InstaSpec V is the company's first air-cooled fibre-optic-coupled ICCD. It is



LOT Oriel's intensified CCDs systems.

designed to be compact, with the detector head incorporating an optimized three-stage TE cooler, intensified power supply and gating electronics. The sensor head saves space and requires no bulky cabling, providing easy adaption to any experiment. Scientific-grade CCD sensors offer

high sensitivity, low readout noise and wide dynamic range. The addition of a gated intensifier, with high-speed (5 ns) gating electronics, to such a CCD increases the system's sensitivity, and allows the detection of single-photon events. The ability to turn the intensifier gain 'on' and 'off' allows *ns* shuttering (usually referred to as gating). This capability is useful for performing reaction kinetics studies, or doing lifetime studies.

Reader Enquiry No. 118

PixelVision has recently introduced the SpectraVideo series of **back-illuminated digital CCD (BCCDs) cameras** for scientific, medical and industrial end-use and OEM applications. These high-performance, 16-bit imaging systems are said to offer excellent sensitivity and low-noise throughout the visible, ultraviolet and soft X-ray spectral regions. SpectraVideo cameras are available with 512×512 ; $1,000 \times 1,000$ and $1,100 \times 165$ element CCD sensors and have two-stage Peltier coolers. The readout rates range from 100 Hz to 1 MHz. In addition to the detector head, each camera is delivered with the Windows-based PixelView image-acquisition and processing software, a 16-bit ISA-bus image-acquisition board, a power supply and a software tool kit. Thinned, back-illuminated CCDs should overcome many of performance limitations of conventional front-illuminated CCDs by illuminating and collecting charge through the back surface of the detector, away from polysilicon electrodes that absorb light.

Reader Enquiry No. 119

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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